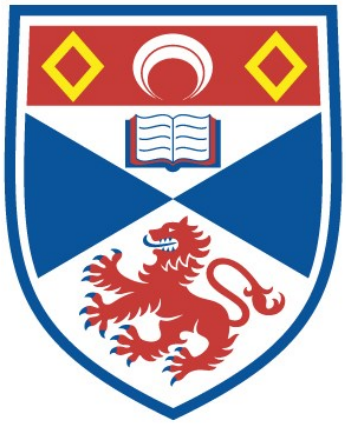
FACTORS AFFECTING EPITHELIAL REGENERATION : WITH SPECIAL REFERENCE TO ASCORBIC ACID AND TO PANTOTHENIC ACID

Nancy Mearns Galloway

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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A THESIS

PRESENTED FOR THE DEGREE

OF

DOCTOR OF PHILOSOPHY

OF

THE UNIVERSITY OF ST. ANDREWS

BY

NANCY MEARNS GALLOWAY, B.Sc.

CERTIFICATE.

I certify that Nancy Mearns Galloway, B.Sc. has spent a total of nine terms on research work first under the direction of Professor R. C. Garry and then under my direction and that she has fulfilled the conditions of Ordinance No. 16 (St. Andrews) so that she is qualified to submit the following Thesis in application for the degree of Ph.D.

Professor of Physiology,
University College,
Dundee.

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Professor of Physiology,
University College,
Dundee.

DECLARATION.

I hereby declare that the following Thesis is a record of results of experiments carried out by me, that the Thesis is my own composition and that it has not been previously presented for a higher degree.

The research work was carried out in the Physiological Laboratories of University College, Dundee, under the direction of Professor R. C. Garry, D.Sc. and of Professor G. H. Bell, M.D.

Signature. Date.

ACADEMIC RECORD.

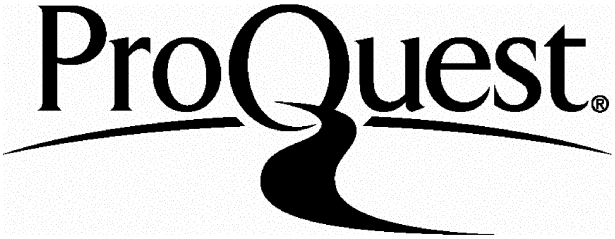
I entered United College, St. Andrews in October, 1942 and in June 1945 graduated with the Degree of Bachelor of Science.

In October, 1945 under the direction of Professor R. C. Garry D.Sc. I commenced the research work which forms the subject of this Thesis. Since October, 1947 Professor G. H. Bell, M.D. has acted as supervisor.

FACTORS AFFECTING EPITHELIAL REGENERATION

with special reference to

ASCORBIC ACID and to PANTOTHENIC ACID.



ASCORBIC ACID

GENERAL INTRODUCTION

GENERAL INTRODUCTION

"It has been frequently stated that the vitamins in general exert an influence on regenerative processes, but until recent years no exact experimental information has been available on which definite opinions might be based."

Arey (1936).

Wound Healing

In most instances all injured tissues regenerate by the growth and organisation of the tissue already present, any differences between wounds in the course of healing being superficial. Hamilton (1889) stated that in clean wounds of adult skin the repair and replacement of epithelium is the dominant feature. The cells, already present, divide and so replace the missing epithelium. Age does not affect this epithelial dominance which appears to be related to the characteristic arrangement of epithelial cells in layers devoid of blood vessels. Arey (1936), in an excellent survey of wound healing, also stresses the important part epithelium plays in all types of repair. In the primary type, with which this work is concerned, the wound is cleft-like and may be healed (a) through simple epithelial regeneration if no other tissues be involved; (b) by direct union of the parts which permits uncomplicated regeneration of epithelium and connective tissue; (c) healing under a scab

which provisionally closes the wound.

In the normal superficial wound in the guinea pig the extension of epithelium from all sides is in progress even on the first day. Two factors are of prime importance in the rapid restoration of epithelial continuity - contraction and cell movement - Arey (1936). These, however, are not the only explanations of wound closure that have been advanced. Some have assigned the whole cause to proliferation (Eberth, 1891; Bardeleben, 1901), while others recognise both mitosis and increase in cell size as minor contributory factors (Werner, 1902; Loeb, 1920). All these factors undoubtedly play a part in the process of healing, but final restoration of epithelial defects is brought about by the movement of epithelial tissue from the intact border zone over the connective tissue or other substrate. Advancing from all sides these modified, reparative epithelial sheets meet and then return to their normal state of organisation.

Contraction plays an important part in reducing the area of an open wound greater than 10 mm. in diameter; on the other hand, it is negligible or absent in wounds of 10 mm. or less - Carrel (1910). The maximum effect of contraction is found in mobile skin which is loosely attached to the deeper structures and in this instance the contraction affects both epidermis and corium as a unit. This can be clearly

demonstrated on removing a circular disc of skin from the outer aspect of the thigh in the guinea pig or rat. The skin-epidermis and cutis vera can be easily cut away from the underlying aponeurosis and contraction takes place early and rapidly until the wound heals as a small line. In the ear, as an example of a location where the skin is fixed to deeper, rigid structures, contraction is a late phenomenon characteristic of the final period of wound closure Loeb (1898). Burrows (1924) interpreted these latter results on the basis of a secondary contraction of the connective tissue which proliferates early in the floor of the wound; hence such contraction is of the nature of a final act and results from a regression of the cicatrix.

Movement of Cells. The chief biological factor responsible for the extension of epithelium over the denuded area is the amoeboid movement of the neighbouring cells themselves. The sequence is as follows. There is an early epithelialisation through active cell movement, in the absence of cell division, and a later mitotic activity at or beyond the margin of the wound. This phenomenon is widespread in the healing of wounds both in invertebrates and vertebrates. Arey and Covode (1937) state that the rapid repair of wounds of the corneal epithelium shows all indications of being due to an active creeping of the epithelial cells.

Mitosis is not a feature of the initial stages of healing and in small wounds mitotic activity may not show any increase until after the epithelialisation is complete - Arey (1932). In such instances the mitotic region may be quite outside the repair area, and this activity can then be interpreted as compensatory and for the purpose of restoring cells lost to the wound by "emigration." In the study of the repair of corneal epithelium Arey and Covode found an actual decline in mitotic frequency during the first days following an injury. Although wound closure was complete in six to ten hours it was not until after the fourth day that the rate of mitosis returned to normal. This may be followed by a marked acceleration during the fifth day, to exceed the normal rate by 75 per cent, and then the mitotic activity returns to normal again.

Cell Growth. During epithelial repair the cells and their nuclei gain in size both in the old epithelium adjoining the wound and in the new covering layer - (Spain and Loeb, 1916); Akaiwa, 1919). The average gain is about 35 per cent, and this maximum is attained just prior to the closure of the wound. The size increase contributes in a minor way to the forward extension of the growing membrane, besides adding to its thickness - Loeb, 1920. The rate of epithelial movement depends, among other factors, upon the size of wound - "the

rapidity of epithelial growth varies directly with the size of the wound" - (Spain and Loeb, 1916; Carrel and Hartmann, 1916). The epithelial tongues also extend faster in smooth, shallow wounds than in uneven wounds or those in which a firm adherence of the scab presents obstacles to the movements of the cells. This epithelial movement has been found to be most energetic in the guinea pig and much weaker in the rat - Arey, 1936.

Size and Age. The factors of size and age also have potent influences on the rate of healing. AGE - This relation has been investigated with great precision by du Noüy (1932) who has shown that the age of a person can be expressed in terms of a constant. This constant, when used in the formula of the healing rate, corresponds to the physiological activity and age of the subject. Or, stated differently, the index of cicatrisation of a wound indicates exactly the age of a patient. The increased rapidity of healing which is characteristic of a younger individual is usually accepted as being due to an increased rate of cellular proliferation. Another interpretation is given by Howes and Harvey (1932). They believe that healing in the wound is more rapid than in adults because fibroplasia begins earlier and is less retarded. Whatever the true explanation may be, it is obviously important that, in all experiments on the effect of wound-healing substances the animals should be of

the same age.

SIZE. The factor of size plays a part of prime importance in determining the rate of healing of a clean wound and every care must be taken to ensure that the sizes of experimental wounds are equivalent in area when it is desired to compare directly their rates on healing. This precaution is of prime importance when the number of wounds available for comparison is small - (Young, Fisher and Young, 1941). "The rate of cicatrisation is proportional to the area but diminishes less rapidly than the area" - Carrel and Hartmann (1916). Thus, in every experiment the technical procedure of wounding must be accurate and precise to ensure that the sizes of wounds are the same.

Influence of Diet. One factor which has only recently been discovered to have a pronounced effect on wound healing is metabolism. Clark (1919) was the first to study the effect of diet on the course of healing in dogs. The latent period, which follows the injury and during which the wound remains practically the same size, can be prolonged or shortened according to the diet. Clark found in his studies that the sole influence was upon the latent period, whereas the periods of contraction and epithelialisation remained unchanged. Hence the type of diet used influenced the total period of healing proportionately as it affected the latent period. High protein diet eliminated the latent

period completely whereas high fat diet prolonged it to six days. High carbohydrate and mixed diets had an intermediate effect. A complete, adequate diet must consist of Carbohydrate, Fat, Protein, Mineral Salts, Water and Vitamins. When one of these factors is missing metabolism of the body is upset and wound healing is liable to be affected. Howes, Briggs, Shea and Harvey (1933) found that there was a considerable retardation in the rate of return of strength in stomach wounds in young rats on half-adequate diets i.e. when one of the above components of a complete diet was missing.

The vitamins are essential components of a balanced diet for perfect metabolism in the body. They are naturally occurring organic substances which possess unique molecular structures that most organisms are unable to synthesise but which are vital parts of the chemical machinery of the cells - Wolbach and Bessey (1942). Considerable attention has been given to the role of vitamins in regenerative processes. Avitaminoses B and C have both been reported to inhibit normal healing, with resumption of healing following ingestion, or local application, of the specific vitamin involved in the deficiency - Lanman and Ingalls, 1937.

Although for many years it was known that inadequate diets cause disease the existence of vitamins was only recognised at the beginning of this century. The realisation that scurvy, beri-beri and rickets are due to dietary errors took place long before the word "vitamin" had been introduced. One of the earliest and most notable examples of this was James Lind's classic "Treatise on

Scurvy" in 1757, when he gave the first clear account of the disease and established the efficacy of lemon juice for its prevention and cure. Lind also observed that scurvy had an unfavourable influence on the healing of wounds in man. However, it was not until 1912 that an outstanding chemist, Funk, propounded the famous "vitamine" theory. Funk, along with other co-workers, predicted with remarkable accuracy which diseases would prove to be due to "vitamine" deficiency, caused by the absence from the diet of "special substances which are of the nature of organic bases which we will call vitamines." The term "vitamine" was modified by dropping the final "e" at the suggestion of Drummond (1920) when it was learned that an amine group was not a characteristic of "vitamines." The initial work on the vitamins was concerned with fundamental problems dealing with the biological significance, chemical structure and methods of estimation. However the knowledge of the symptoms and diagnosis of vitamin deficiency is largely due to the investigations of workers on the effects of vitamin-free, synthetic diets on animals.

Wolbach and Bessey (1942) point out that a few considerations should be kept in mind in planning vitamin deficiency experiments either for the purpose of characterisation of a deficiency or for the elucidation of normal processes of growth and function. One is that the dietary regimen should be optimal in every respect excepting the content of the vitamin concerned, because other conditions which retard the general metabolic rate may decrease that vitamin requirement. A second is that the consequences of a complete deficiency may be so severe as to prevent survival of the animal for a sufficient length of time for the development of distinctive, functional and structural changes, at least in demonstrable form. A third is that the more rapid the growth rate the greater is the effect of vitamin lack upon the processes concerned in the synthesis of structural

materials, e.g. formation of collagen in ascorbic acid deficiency. A fourth is that there are important species variations and ascorbic acid deficiency is a good example of this again. Most vertebrates, such as the rat, pigeon, etc., are completely independent of outside sources of the vitamin while, on the other hand, an adequate intake is essential to the life of the guinea pig, monkey and man.

Ishido (1923) fed guinea pigs and rats on a completely vitamin-free diet and reported that they showed a plain tendency toward delayed healing of incised wounds in comparison both to normally nourished animals and to those underfed on a normal diet. In the individuals suffering from total vitamin deficiency the adhesion of the walls of the wound was faulty and there was a tendency toward a defective new formation of connective tissue. Moreover, fluid collected in the floor of the wound while cavity formation, necroses and susceptibility to infection were additional characteristics. One wound did heal in such an animal but it required twice as long as the normal cicatrisation period. These results are in accord with similar experiments carried out by Lauber (1934) on the rat and guinea pig. Animals subjected to a vitamin-free diet always showed delayed healing of incised wounds. Lauber suggests that, as for hormones, the favourable effect of vitamins is accomplished through altered cellular metabolism.

Moreover, both vitamins and hormones are considered to belong to the same biological category, their respective representatives having both synergistic and antagonistic relations to each other. Saitta (1929, 1930) reported that certain avitaminoses - B and C notably - greatly diminished the power of cicatrisation in incised wounds in rats and guinea pigs but on oral administration of the vitamin, or on direct application of an extract of the vitamin, cellular repair was activated so that healing occurred in a shorter period than normal. These results have been opposed by several workers including Lauber (1934) and Larcher (1927). No definite conclusion between these conflicting findings could be reached at that time, due to the lack of standardisation of the exact amounts of the vitamin administered. The claims of Saitta tend to include the vitamins in the class of "growth-promoting" or "wound-healing" substances which have recently had so much importance attached to them. Such a substance, according to Young, Fisher and Young (1941), is specifically designed to impose a new, quick and artificial rhythm (exceeding the "normal" rhythm) on the processes of cell division of the fibroblasts and other reparative elements in a healing wound. However it is not sufficient that a wound-healing substance accelerate the rate of healing. It must promote sound and durable healing of a wound. Soundness of healing can only be established by careful inspection over

a long period after epithelialisation and cicatrisation have been completed. There is often a lag in the terminal stages of healing and if this occurs in a wound treated by a wound-healing substance any initial acceleration produced by the substance may be nullified in the later stages. Even a more serious effect can be conceived whereby the reparative elements of a healthy, granulating wound, possessing a natural and normal impulse to proliferate, might acquire a resistance to the growth-promoting substance and cease to proliferate altogether - a healthy wound may be transferred into an indolent one. In the biological assay of an alleged wound-healing substance, experimental wounds in animals serve an essential but not a definitive purpose. Young, Fisher and Young (1941) state that such wounds can decide questions of the following order; (a) whether the substances are able to accelerate the process of "normal" healing of clean and healthy wounds to a significant extent without impairing the soundness of healing, and, if so, (b) whether the acceleration is uniformly sustained throughout the process of healing or (c) whether a retardation of healing or a tissue resistance develops at any stage, followed perhaps by a phase of greater acceleration, and (d) if a retardation develops at any stage, whether it can be minimised or eliminated by any readjustment of the intervals of application or of the concentration of the agent. If the results of a wound-healing substance,

which has been attested on experimental wounds in animals, prove to be favourable, then the definitive test of the human therapeutic efficacy of the substance must be carried out on wounds in the human subject before complete evidence of its use in the clinic can be attained. However any substance which is suspected of activating the process of reparation in lower animals must be definitely given due consideration, as the development of methods for the stimulation of growth of cells would greatly improve the therapeutics of human skin lesions.

With advancing knowledge of the chemical nature and structure of the vitamin molecules it has been shown that many of these appear to take part in the oxidation-reduction processes which constitute the metabolism of tissue cells. Their relation to oxidation and reduction also indicates that they may be directly concerned in the maintenance of normal behaviour of tissue cells and that in many cases the specific symptoms resulting from a prolonged inadequacy of these factors may be merely the result of disturbance of metabolism in one or more tissues - Eddy and Dalldorf (1941). Ascorbic acid (Vitamin C) and Pantothenic acid are both known to function in some fundamental manner in the metabolism of all living matter - Wright (1942).

ASCORBIC ACID AND SCURVY

One of the functions of ascorbic acid that has been

fairly well established is its role in the formation of intercellular substance in the animal organism. The deficiency does not prevent the multiplication of the cells of each type of tissue, but the cells assume a common morphologic character, approximately that of embryonic connective tissue, and the histologic appearance indicates that a liquid material is produced in lieu of normal matrix - Wolbach (1936). In the older medical writings there were many references to the breaking open of old wounds and the refracturing of old fractures in scorbutic persons. Bleeding gums, loosened teeth and petechial haemorrhages are often mentioned in the early papers on scurvy. Although the disease, scurvy, has been a menace to seafarers, explorers and armies since classical times, it was not until the beginning of the twentieth century that the pathology of scurvy was extensively studied. Hőlst and Frölich (1907) were the first to produce experimental scurvy in guinea pigs. They showed that a condition of scurvy, similar to that of the human being, could be produced in guinea pigs if they were fed solely on a diet of oats and bran. This was followed by the work of Hart and Lessing (1913) who produced experimental scurvy in monkeys. In 1919 Aschoff and Koch published an account of the microscopic pathology of scurvy and in 1924 Höjer's comprehensive work on the histology of experimental scurvy appeared. These two

papers confirmed the fact that, from a pathogenic point of view, guinea pig and human scurvy have many points in common. Funk (1912), in his first review of possible avitaminoses, suggested that scurvy might be a vitamin deficiency and from this suggestion modern research on this vitamin was initiated. However it was almost twenty years later before its structural formula, nature and properties were discovered. The characteristic of the vitamin that proved the best clue to its nature was its instability. In the early attempts to isolate the vitamin from lemon juice it became increasingly evident that oxidation rapidly destroyed the potency of the vitamin - Zilva, 1928. The first definite identification of vitamin C was reported by Waugh and King (1932), followed in a short time by confirmatory evidence from Svirbely and Szent-Gyorgyi (1932). After finding that it was a lactone of an acid derived from a hexose sugar, Herbert, Percival, Hirst and co-workers (1933) established its structural formula. In the same year, Szent-Gyorgyi and Haworth gave the name ascorbic acid to the vitamin to denote its antiscorbutic action - the original term hexuronic acid being superseded. It is now known that vitamin C is synthesised by all the higher plants and by many animals except the primates, including man, the guinea pig and some of the ungulates. The guinea pig has thus become the most important animal in the studies of experimental scurvy as it is very susceptible to the disease.

The usual pictures of a scorbutic guinea pig are a crouched position, in which the animal may be seen raising its tender and inflamed limbs off the ground to keep pressure off them, and a "face-ache" position in which the animal lies on its side to ease its inflamed jaw - Harris (1935). In guinea pig experiments, reported in 1931 by Dalldorf, it was stated that animals placed on a vitamin C free diet developed capillary fragility which did not develop regularly but was subject to a transient reversal during the early stages of depletion. In other words processes of adjustment or compensation were probably present; this view has since been confirmed - Giroud (1935); Wacholder (1938). One of the first manifestations of the deficiency in guinea pigs is rapid wasting which develops within the second week and most animals die at the end of the third week on completely ascorbic acid deficient diets. On partially deficient diets the animals live for months and develop the lesions long associated with scurvy in man. The papers published by Aschoff and Koch (1919) and by Höjer (1924) on the histology of experimental scurvy characterised it as a condition in which defective intercellular materials were formed. These results showed that, from a pathogenic point of view, guinea pig and human scurvy have many points in common. Wolbach (1937) summarised this fact in his statement that "the gross and microscopic pathologic changes of human scurvy as seen in the infant, and

experimental scurvy as seen in the guinea pig, are so nearly identical that no reasonable doubt can be entertained with regard to applying to the human being the facts ascertained from the experimental studies." As one example of this fact, Göthlin (1934) found that the stage of scurvy in guinea pigs which is recognisable only by means of microscopic alterations in the teeth is equivalent to that prescorbutic stage in man which is indicated by fragility of the capillaries. However, as Stefansson (1939) has pointed out, too much reliance ought not to be placed on guinea pig experiments. These may not be directly applicable to man. Requirements of the vitamin for man, estimated from guinea pig experiments, may be two to ten times too great - probably man can adapt to a low vitamin intake. In contrast it must be remembered that the morbid effects of vitamin C deficiency are best studied in experimental animals where complete dietary control permits the examination of lesions at all stages of the disease. Only from these observations can the explanation of the pathogenesis of scurvy be applied to human cases of the disease.

The outstanding feature in the production of the pathology of ascorbic acid deficiency is failure in the formation of intercellular materials. Wolbach and his associates in a series of papers from 1926 to 1942 showed that reticulum and collagen are not formed in the scorbutic animal. Normally in the intercellular substance fibroblasts lie in an amorphous

ground substance within which fibrils of a reticulum are found as wavy bands of collagen - Bicknell and Prescott (1942). The fibrils are cemented together by a translucent matrix. In the scorbutic animal the ground substance and fibroblasts are present but no collagen is formed. After administration of antiscorbutics intercellular fibrils are formed in considerable amounts within twenty-four hours. Formation of such fibrils occurs in regions where mesenchymal cells have accumulated in continuation of normal growth activities, in repair of spontaneous lesions in consequence of scorbutus and in the organisation of blood clots after excision of tissue. Furthermore, collagen formation occurs where reparative cellular responses are most active - Wolbach (1933).

The role of vitamin C in the formation of intercellular materials in the human subject has been established without doubt by the work of Hunt (1940) and of Crandon (1940). Crandon found that although healing appeared to progress in an incised skin wound, in a scorbutic person, there was no healing of the wound beneath the skin which was filled with an unorganised blood clot. Keratosis of hair follicles and other lesions of the epidermis were also observed by Crandon in the human subject on an ascorbic acid-free diet, supplemented with all "other known vitamins," which seems to prove that the epidermis is affected, particularly when such lesions disappeared after ascorbic acid therapy. Capillary

bleeding is also common in scurvy and is probably the result of structural weakness, either the result of changes in the cement substance binding the endothelial cells together, or in collagen fibrils immediately adjacent to the capillaries - Dalldorf (1939). Other lesions characteristic of scurvy occur in the teeth and long bones. In these structures the parenchymal cells, whether odontoblasts or osteoblasts form fluid instead of the normal substantial dentine or bone, or, in cases of partial deprivation they form defective materials which, while solid, are distinctly inferior. The result of this fundamental change is that teeth and bones cease to grow, and become gradually more porotic and fragile.

Since vitamin C is essential for the formation of intercellular material it is necessary for wound repair. Lanman and Ingalls (1937), Taffel and Harvey (1938) have shown that the tensile strength of healing incised wounds in guinea pigs, suffering from a partial deficiency of vitamin C, is considerably less than in normal animals. Wounds from operative incisions ruptured at a pressure of only a third of that required to rupture wounds of normal animals. The scar tissue was also distinctly abnormal; there was a marked decrease in the intercellular material and a disorganised arrangement of the fibroblasts. Hunt (1941) also observed that such scars were "puckered, stretched, sunken, loose,

irregular and almost avascular." It has also been observed that, on a high intake of vitamin C, the tissues of the healing area around a wound contain more vitamin C than those from normal controls; if the organism is on a scorbutic diet there is no increase in vitamin C in the healing area - Bartlett, Jones and Ryan (1942). Bourne (1944), in accordance with this fact, found that with animals on a low vitamin C diet, the organs lose most of the vitamin when the animal is wounded and the vitamin can be demonstrated in the tissues surrounding the wound. Presumably there is an increased demand and mobilisation of vitamin C for the healing process. The more extensive the injury the more vitamin C required - other organs would become deficient and this suggests that a routine administration is desired. It is not known whether a state of saturation is necessary for the most rapid and effective deposition of collagen or whether a vitamin content far short of saturation is enough. Bourne (1944) stated that as far as guinea pigs are concerned, saturation with vitamin C is not an essential prerequisite of optimum healing. A subscorbutic state has been reported in guinea pigs by Mouriquand (1938), who showed that the animals can become weak, emaciated and finally die after living on diets low in vitamin C without showing frank signs of scurvy. A state of vitamin C deficiency without the clinical manifestations of scurvy has

also been reported in the human subject. This degree of deficiency, or asymptomatic scurvy, may well have an important bearing on wound healing. It could be the fundamental reason for many indolent wounds not exhibiting the processes of normal healing.

In spite of the large amount of work done on the influence of ascorbic acid deficiency on wound healing, no clear differentiation has been made between wounds involving all tissues and wounds chiefly of the epithelium. The experiments so far carried out do not differentiate between the action of vitamin C on repair of connective as contrasted with epithelial tissues. Thus, in view of the important part ascorbic acid is now known to play in the regeneration of connective tissue, it seemed most desirable to discover whether vitamin C affected in a similar manner the regeneration of epithelial layers. For this reason the stratified epithelium covering the cornea, the muco-periosteum of the gum, and the skin on the ear and thigh of guinea pigs and rats were chosen as sites for the experimental wounds.

PANTOTHENIC ACID

This vitamin is one of the most recent to be discovered and much of its chemistry and physiology still remains unknown. Pantothenic acid was first recognised as an essential nutriment for certain strains of yeast by Williams, Holady and co-workers

in 1933. Williams (1939) isolated this important factor and found it to be a member of the vitamin B complex group and with Major in 1940 determined its structure. The role played by Pantothenic acid is without doubt a fundamental one since it appears to be present in every living cell. In a complex organism it is essential to all types of cells, and to the functioning of all kinds of tissues. It is not surprising in view of this fact that diverse pathological changes may result from its deficiency - Williams (1943). Human muscle has been found to be richer in pantothenic acid than rat muscle (although not in other B vitamins), hence having a better resistance to deficiency. It seems probably that the distribution of pantothenic acid in the tissues of various species has an important bearing upon the question of how different animals react to this deficiency. It was found that pantothenic acid is identical with one of the filtrate factors necessary for the growth of rats - Macrae and co-workers (1939) - and a year later it was identified with the anti-grey hair factor - Györgyi and Poling (1940); Unna and Sampson (1940) (1941). Pantothenic acid has also been identified as the chick antidermatitis factor and lesions in the marrow and peripheral nerves of hens have been observed to regress as soon as this vitamin was administered. However it is in rats that the most severe symptoms of the deficiency have been observed.

Rats maintained on a diet devoid of pantothenic acid cease to grow in three to four weeks and develop a deficiency syndrome characterised by scant, coarse fur, inflammation of the nose and blood-caked whiskers - Unna (1940). The so-called blood is in reality a porphyrin deposit - McElroy (1941). Loss of weight, paralysis of the hind legs, a hyperkeratotic, atrophic and desquamative dermatosis and myelin degeneration of the sciatic nerves and spinal cord have also been described in mice fed on a deficient diet - Sieve (1941). Daft and Sebrell (1939) found that there also occurred in deficient rats haemorrhage, atrophy and necrosis of the adrenals and testicular degeneration. The most conspicuous external evidences of deficiency noted are an affection of the eyelid, which disturbs the eyes in some cases to such an extent that they cannot be opened, "spectacled eyes," coarse, lifeless, grey hair and, in some cases, alopecia develops behind the fore limbs on the chest and across the back - Carter (1945); Wooley (1941); Henderson (1942). Changes in the oral epithelium have also been observed. Wainwright and Nelson (1945) found that there is an early hyperkeratosis of enamel of the teeth and of the mouth epithelium. There is a progressive necrosis of the oral epithelium and subsequent destruction of the underlying connective tissues - osteoporosis and progressive marginal atrophy of the alveolar bone were also found in several cases. An interesting point, which is

difficult to explain, is the unusual absence of inflammatory response, notwithstanding the presence of marked destruction of tissue - Sullivan and Nichols (1942).

The pantothenic acid requirement for the maintenance of optimal growth in rats has been the subject of much controversy. Unna and Richards (1942) report that the dose required to prevent the development of characteristic lesions is as high as 100 micrograms per day at three weeks of age. Henderson and co-workers (1942) found that 40 micrograms daily prevented or cured nutritional grey hair in rats while Schwarz (1944), in his experiments on the effect of pantothenic acid on the growth of rats, found that 20 micrograms prevented loss of pigment and only 5 micrograms daily prevented complete cessation of growth. As this subject is still in the preliminary stages of investigation a definite result will no doubt be reached in due time. It has been proved, however, by Unna and Creslin (1940), Spies, Hightower and Hubbard(1940) that pantothenic acid is, like other members of the vitamin B complex group, a substance of extremely low toxicity. Doses as high as 10 grams of calcium pantothenate per kg. administered to rats by mouth had no toxic effects. The effect of large doses was studied in young rats fed 50 mg. and 200 mg. of calcium pantothenate per rat per day over a period of 120 days. The animals developed normally, their growth rate did not differ significantly from that of the control

group. The pantothenic acid content of the blood was increased soon after administration of the vitamin but returned to its previous level within twenty-four hours - excessive amount recovered in the urine. It can thus be stated that as long as the minimum quantity of pantothenic acid is administered to the animal daily, there is no fear of excessive quantities of the substance doing any harm.

The use of pantothenic acid in therapeutics is relatively recent and little known in contrast to the numerous observations on avitaminotic symptoms. One of the few applications comes from Sciclounoff and Naz (1945), who, in several clinical observations, used pantothenic acid in the form of "Bepanthene-Roche" in an ointment, jelly or aqueous solution on ulcers of the skin. These different preparations were well tolerated by the tissues and the pantothenic acid showed itself to be active against infected sores of the skin and mucosa. The result of these researches, made on the action of "Bepanthene" in local application, permitted these workers to conclude that this vitamin product possesses the property of stimulating the repair of injured and gravely infected tissue. Although no clinical sign pointing to a deficiency of pantothenic acid has been observed in man, Spies (1940) believes such a thing can exist. Spies found that patients with an abnormally low concentration of pantothenic acid in the blood also had less than normal in the urine. These observations suggest that

pantothenic acid is important in human nutrition. It may be the case that the patients treated with "Bepanthene" had a low concentration of the vitamin in the blood and thus responded to the treatment very favourably. This may be true, but if such a supposition is to be accepted clinically, it must first have experimental proof from detailed investigations. With this view in mind the work on pantothenic acid in this thesis was carried out in an attempt to find the answer to this "questionnaire."

ASCORBIC ACID

SECTION A.

SECTION A

1. THE INFLUENCE OF ASCORBIC ACID ON HEALING OF SKIN WOUNDS IN RATS

Introduction

Oral Administration

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Direct Application

(a) Methods

(b) Results

2. THE RATE OF HEALING OF SECOND AND SUBSEQUENT WOUNDS IN RATS

Introduction

Methods

Results

1. THE INFLUENCE OF ASCORBIC ACID ON THE HEALING OF SKIN WOUNDS IN RATS

Introduction

Although "rat scurvy" was first reported in 1921 by Shipley, McCollum and Simmonds, the diagnosis was not proven. Several other workers, including Vedder and Rosenberg (1938), reported that they could produce scurvy-like symptoms in rats and that 0.5 mgm. of ascorbic acid protected the animals "almost fully." However, Parsons and Hutton (1924) and Lepkovsky and Nelson (1924) found that the livers of rats reared on a diet deficient in the antiscorbutic factor contained significant amounts of this factor. A few years later Guha (1935) found that certain tissues of the rat, including the spleen, kidney and liver, are able to form significant amounts of ascorbic acid. They do so by converting mannose into ascorbic acid, but the ability with which the isolated tissues can carry out the conversion differs considerably according to the species.

However, although it has been postulated many times that rats can synthesise this vitamin and are thus independent of an external source, it must be remembered that Horden and Zilva (1918) found rats grew better if ascorbic acid was supplied, and this may still be fundamentally true. Excess ascorbic acid does not have any effect in accelerating the regeneration of bone in rats, according to Bourne (1942), but

there are no results to show whether regeneration of epithelium in rats differs in any way from tissues of mesenchyme origin when there is an excess of the vitamin present.

ORAL ADMINISTRATION OF ASCORBIC ACID

(a) Methods

Rats of the same age, almost mature, and off the same strain were selected for the experiments. The rats were divided into two groups, (a) Controls, each of which received 25 mgm. ascorbic acid in 10 c.c. milk every day, and (b) Principals which received no ascorbic acid. The rats were all kept on a stock diet of rat cubes (Thomson, 1936), ascorbic acid-free. There was an initial loss in weight in both groups, but this was probably due to an adjustment to diet since both groups quickly regained their initial weight. The animals were kept on this diet for approximately forty-eight days before being wounded in each thigh (v. infra.)

At intervals, during this period, titrations of the amount of ascorbic acid in the urine of both groups of rats were carried out by the 2:6 dichlorophenolindophenol method, as described by Harris and Abbasy (1937). It was found that the urine, on standing, quickly destroyed any ascorbic acid present. However, if fresh urine were used, significant positive results were obtained. Average results with urine titrated within one hour of passing were:-

Rats with ascorbic acid supplement..24.2 mgm./100 c.c. urine. Rats with no ascorbic acid supplement 5.3 mgm./100 c.c. urine. Thus it may be said then that the controls had a luxus consumpt of ascorbic acid, and this led to the question as to whether flooding of the rat tissues with ascorbic acid would influence the repair of epithelium.

A number of pilot experiments, four pairs of rats being used, were carried out at first and the results obtained were confirmed in a major experiment. Special care was taken to ensure that all rats were of the same age and that the sizes of the experimental wounds were equivalent in area, as these are important factors in wound healing (v. supra.)

Cuthbertson, Shaw and Young (1941) described a method for removing circular sections of the skin in rats and from this the following technique was evolved.

Twelve mature Controls and twelve Principals were used. The animals were first anaesthetised with ether and the hair closely removed with hair clippers from the dorsi-lateral aspect of each thigh. To avoid irritation of the skin, shaving, depilation by plucking or chemical preparations, such as barium sulphide, were not used. A demarcation on each thigh was made with a specially sharpened cork borer, of diameter 12 mm., and the areas were carefully cut out with scissors, taking the minimum of subcutaneous tissue.

In the operation, no actual incision was made with the borer, as the underlying muscle is easily injured and this must be avoided. Rarely any bleeding was observed and no antiseptic or dressing was applied. The animals appeared to be undisturbed by the operation and did not lick their wounds which quickly dried up. Sepsis was observed in none of the wounds, despite the fact that the only precaution taken to prevent it was the sterilisation of all instruments before wounding.

Histological sections were made of the excised disc of skin to determine the exact amount of epidermis removed. - Fig. 2. Portions of skin are notoriously difficult to keep flat and to prevent from curling during fixation. The following technique was therefore devised. The disc of skin, immediately after removal, was carefully spread flat between two circular sheets of filter paper. These filter paper circles just fitted into a Petri dish. A second Petri dish, of slightly smaller diameter, was placed on top of the filter papers. Fixative was then run in between the two Petri dishes for the usual time of fixation. Thereafter the normal method of dehydration and embedding was employed (v. infra.) By the use of this method and not the ones suggested by Carleton (1938) and Cowdry (1943), in which the skin is either pinned out on a piece of cork or spread down on a wooden tongue depressor for the first few minutes of

Figs. 1 and 2.

Fig. 1. shows diagrammatically how the excised disc of skin was spread flat between two circular sheets of filter paper. These filter paper circles fitted into the outer Petri dish and the inner Petri dish, of slightly smaller diameter, placed on top of the filter papers. The section of skin was thus unable to curl in at the edges and after fixation, a perfectly flat piece of tissue was obtained.

Fig. 2. shows the exact amount of skin removed. - epidermis and cutis vera. No muscular tissue was cut away. (X 90)

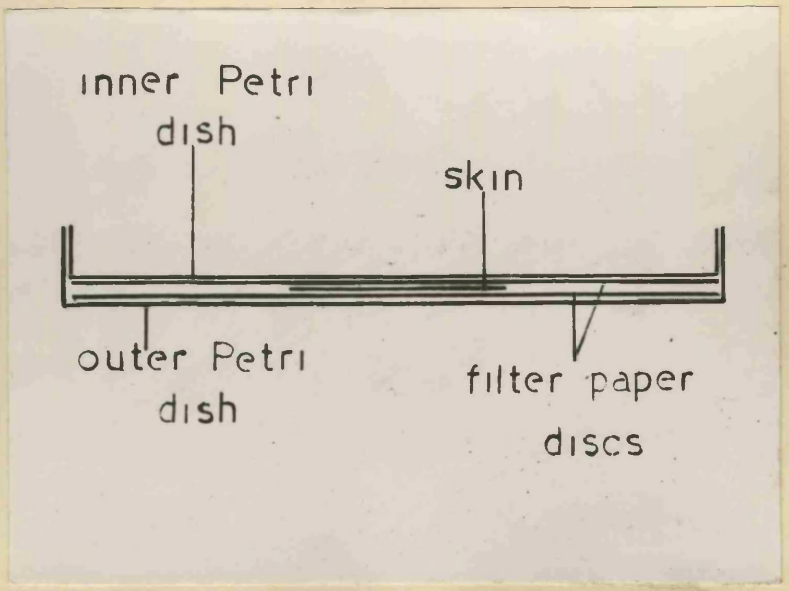

FIGURE 1

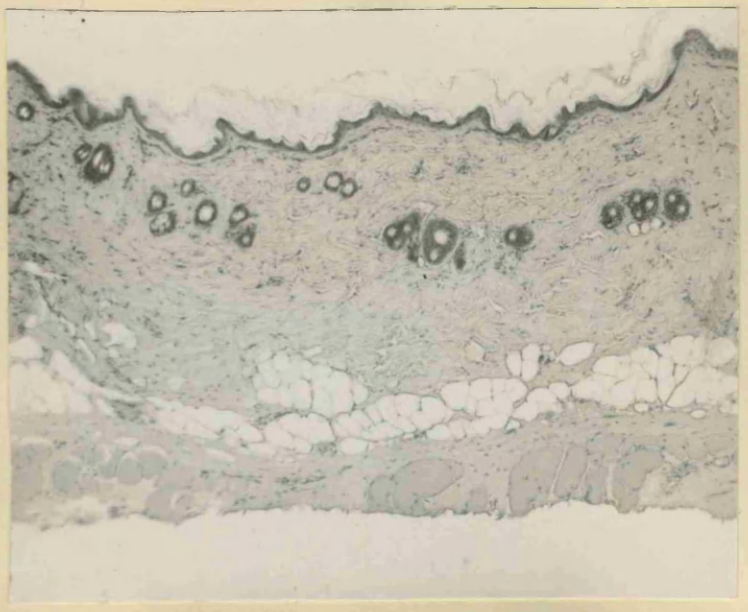

FIGURE 2

fixation, the skin does not tend to curl in at the edges and a perfectly smooth piece of tissue is obtained as shown in Figs. 1 and 2.

Histological sections were also made of the healed areas and underlying tissues to confirm by microscopical observation the results obtained macroscopically. Good sections of skin and underlying tissues are very difficult to obtain as in the preliminary processes of fixation and dehydration, the varying tissues tend to harden to very different degrees.

The choice of fixative is very important. There is so much controversy in histological literature about the best fixative for skin, that it was essential at first to try out various solutions in order to select the one which gave the best results. From the following fixatives - Bouin, 4 per cent Formol, Mercuric formol, a mixture of Mercuric chloride, Formalin and Acetic acid, Picric acid dioxane and Absolute alcohol - it was found that Mercuric formol was the most successful. Consequently in all histological work on the skin, this fixative was used made up in the proportion:-

90 c.c. saturated Mercuric formol
10 c.c. 40 per cent formaldehyde

Best results were obtained by perfusion of the entire hind quarters of the animal with fixative through the abdominal aorta. Mercuric formol was allowed to flow through for half

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an hour, after Ringer had previously washed out the blood. This method gave a quick, uniform and thorough fixation. Without such preliminary fixation of the entire hind quarters it was practically impossible to excise the wounded skin area and underlying tissues in one block, so loose is the connective tissue binding skin to underlying structures that the skin inevitably separated. The piece of tissue was now excised, placed in fixative overnight and then for four days in 4 per cent phenol, which has a marked softening quality without any special impairment of staining qualities.

The tissue was now dehydrated in alcohol and this process also required care, as alcohol tends to make skin very hard. It has been advised to cut the time of dehydration to a minimum, but, although a short period may be suitable for a thin piece of tissue, it was found that with tissues 5 mm. or more thick, the dehydration was not complete. At first, the technique of passing the fixed tissue through the different grades of ethyl alcohol, 30 per cent, 50 per cent, 75 per cent and then butyl alcohol - twenty-four hours in each - was attempted, but not very satisfactory results were obtained. After several experimental trials, the following mixtures proved to be very satisfactory, and this form of dehydration was used in all future work. Embedding was always done in pure paraffin.

1. 45 per cent ... Ethyl alcohol ... 90 c.c. ... 24 hours
Butyl alcohol ... 10 c.c.

2. 70 per cent ... Ethyl alcohol ... 75 c.c. ... 24 hours
Butyl alcohol ... 25 c.c.

3. 90 per cent ... Ethyl alcohol ... 45 c.c. ... 24 hours
Butyl alcohol ... 55 c.c.

4. 100 per cent ... Ethyl alcohol ... 25 c.c. ... 24 hours
Butyl alcohol ... 75 c.c.

5. Three changes in Butyl alcohol ... 24 hours each

6. One hour in Butyl paraffin

7. Twenty-four hours in pure paraffin

The paraffin blocks were now placed several days in "Mollifex" - a solution for softening hard tissues - after which very fine sections could be cut at approximately 8µ on the Cambridge microtome.

Ehrlich's acid Haematoxylin and Orange G were used to stain all sections.

The criteria of healing were the disappearance of all granulation tissue and complete epithelialisation of the wounded area (macroscopic observation).

(b) Results - The effect of "Luxus consumption" of ascorbic acid by mouth in rats.

After the discs of skin were removed, the wound subsequently enlarged, as a result of taking the disc from a relatively elastic and mobile skin, but remained circular in outline. It was interesting to notice that once epithelial regeneration commenced, the wound healed as an ellipse, until by the final day it ended as a very small, narrow line.

There proved to be no statistical significance between the two groups, as seen from Table I, the Controls all being healed in an average of 15 days and the Principals in 16 days.

TABLE I

Effect of "Luxus" consumption of Ascorbic acid by mouth in rats.

Controls:- Standard diet, with 25 mg. Ascorbic acid by mouth daily in solution. 12 animals, with bilateral wounds - 24 wounds which healed in 15 days.

Principals:- Standard diet, no additional Ascorbic acid. 12 animals with bilateral wounds - 24 wounds which healed in 16 days.

The formula recommended by Burn (1937) $\frac{m_1 - m_2}{\sqrt{E_1^2 + E_2^2}}$ was used to determine the statistical significance.

No. of Wounds	Mean Period required for healing		Difference between Controls and Principals DAYS
	Days	± S.E. of mean	
24 C	15	± 0.66	1
24 P	16	± 0.33	

m = mean period for healing
E = Standard error S.E.
C = Controls
P = Principals

$$\frac{m_p - m_c}{\sqrt{E_p^2 + E_c^2}} = \frac{1}{0.74} = 1.33$$

Only if the ratio $\frac{m_1 - m_2}{\sqrt{E_1^2 + E_2^2}}$ is greater than 3 can the difference between two series be considered significant.

(c) Histological Picture

One rat from each series, Control and Principal, was killed on the eighth day after healing and fixed by perfusion. A small square of skin, including the wound, was cut out with a thin layer of muscle and placed in the fixative overnight. On examination of the sections cut, it was found that there was no histological difference between the wounds of the two series at this point as shown in Figs. 3 and 4.

The above histological process was repeated in another Control and Principal two and a half months after healing took place and again there was no microscopic difference in the two series as shown in Figs. 5 and 6.

This microscopic observation proves conclusively that the oral administration of excess ascorbic acid does not influence the rate of healing of skin wounds in any way in rats, nor does it seem to affect the histological appearance of the scar tissue.

Figs. 3 and 4

Fig. 3 shows a section of Control rat's skin on the eighth day after healing. No sign of any defect in epithelium or underlying connective tissue layers can be seen. There is no sign of the wound and the skin is quite normal. (X 90)

Fig. 4 shows a section of Principal rat's skin on the eighth day after healing. The histological appearance of this section is identical with the Control's picture and Ascorbic acid deficiency has made no affect on the healing of the skin wound. (X 90)

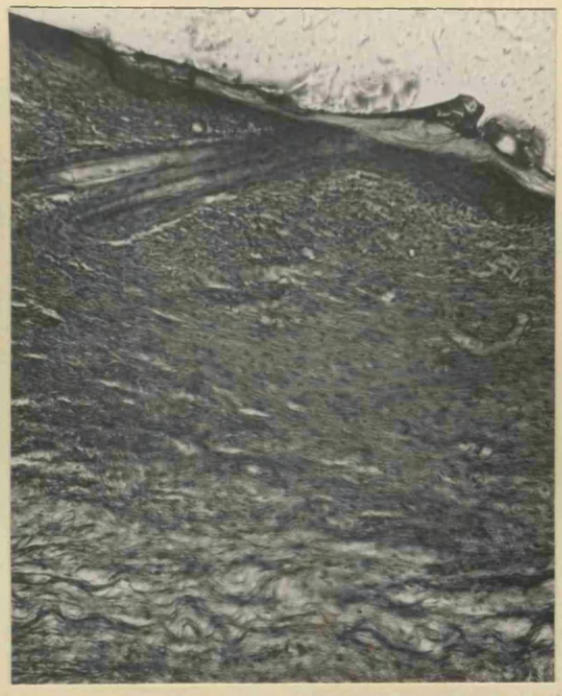

FIGURE 3

FIGURE 4

Figs. 5 and 6

Fig. 5 shows a section of Control rat's skin two and a half months after healing. Both epithelium and connective tissue are perfectly normal. (X 90)

Fig. 6 shows a section of Principal rat's skin two and a half months after healing. Again it appears exactly similar to the Control's picture and all tissues are normal. (X90)

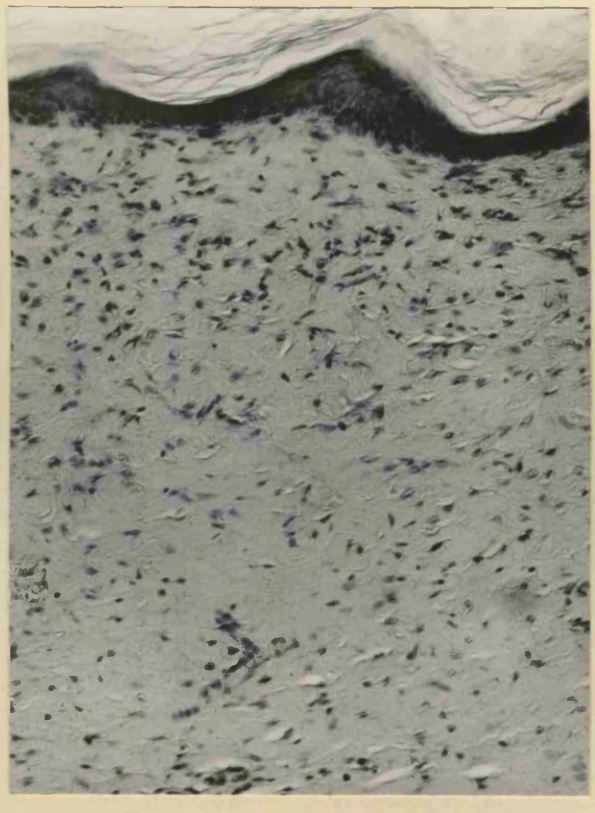

FIGURE 5

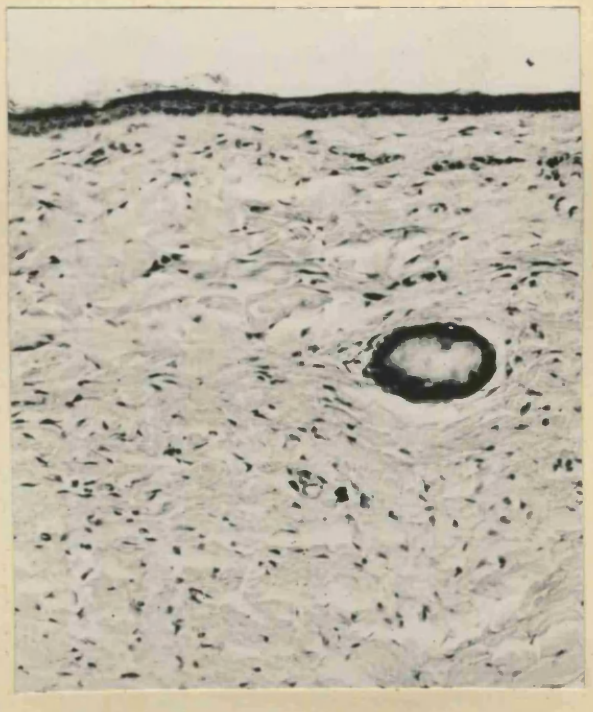

FIGURE 6

DIRECT APPLICATION OF ASCORBIC ACID

The effect of dusting sterilised ascorbic acid powder directly on to skin wounds was now investigated. The same method of wounding the thigh was used in the following experiments as in (a) above.

(a) Methods

A control series was run in conjunction with a principal series, 12 young rats in each. The control rats had their wounds left untouched and the principals' wounds were dusted daily with the powder. The diet in each case consisted of only rat cake-nuts and water.

(b) Results

There was a significant difference in the healing rates.

The wounds of the controls in the earlier stages of healing were less red, thick, and showed less surrounding induration than those of the principals. The scabs of the controls came off earlier and were much more compact and uniform throughout. The controls were healed in an average of 11 days, while the Principals took 18 days. (The difference in healing time of the Controls, as compared to the previous experiment, may be ascribed to the age of the rats. In the previous experiment the rats were at least six months older than the rats in this experiment.)

There was little doubt that this delay was due to the pH of the acid, as it has been proved before that hydrogen ion

TABLE 2

Effect of direct application of Ascorbic acid powder on skin wounds in rats.

Controls:- Standard diet, wounds left untouched. No Ascorbic acid. 12 animals with bilateral wounds - 24 wounds. Wounds healed in average 11 days.

Principals:- Standard diet, wounds dusted daily with Ascorbic acid powder. 12 animals with bilateral wounds - 24 wounds. Wounds healed in 18 days.

No. of Wounds	Mean Period required for healing		Difference between Controls Principals DAYS
	Days	± S.E. of Mean	
24 C	11	± 1.03	7
24 P	18	± 0.76	

$$\frac{m_p - m_c}{\sqrt{E_p^2 + E_c^2}} = \frac{7}{1.28} = 5.4$$

This Table shows that there is quite a significant statistical difference between the two groups.

concentration has a definite effect on wound healing - Davidson (1945). Ascorbic acid, in concentrated solution such as probably existed in the wound, has a pH in the neighbourhood of 3 or 4. Table 2 shows that there is quite a significant statistical difference between the two groups.

In order further to investigate the effect of pH, bilateral wounds were made on 24 rats as before and divided into three groups. In group (A) one wound was painted daily with a solution of sodium ascorbate, the pH being regulated to approximately 6.6 by mixing 2 c.c. (100 mg./c.c.) plus 18 c.c. phosphate Ringer. The other wound was painted with Ringer only, pH approximately 6.6. In other words, the only difference between the two solutions was in the content of Ascorbic acid, osmotic pressure and pH being similar. Both solutions had a solute concentration of about 1 per cent. In group (B) both wounds were painted with Ringer only and in group (C) both wounds were treated with the sodium ascorbate solution. The results, as shown in Table 3, show no significant difference in the rate of healing in the three series.

A final experiment was carried out using a stronger solution of sodium ascorbate applied directly to the wounds. Twelve rats had Ringer only and twelve had 3 per cent sodium ascorbate alone painted on their wounds. In the end all wounds healed up in exactly the same time in both series,

TABLE 3

Effect of a solution of sodium ascorbate, pH regulated to approximately 6.6, on skin wounds in rats.

24 rats divided into three groups.

(A) Standard diet, 8 rats with bilateral wounds - 16 wounds. One wound painted daily with a solution of sodium ascorbate, and the other wound painted with Ringer only.

(B) Standard diet, 8 rats with bilateral wounds - 16 wounds. Both wounds painted with Ringer only.

(C) Standard diet, 8 rats with bilateral wounds - 16 wounds. Both wounds treated with sodium ascorbate solution.

Group A healed in 15 days.

Group B healed in 15 days.

Group C healed in 14 days.

No. of Wounds	Mean Period required for healing		Difference between Groups DAYS
	Days	± S.E. of mean	
16 A	15	± 0.66	1
16 B			
16 C	14	± 0.33	

E_1 = S.E. of (A or B)

E_2 = S.E. of C

$$\frac{m_2 - m_1}{\sqrt{E_2^2 + E_1^2}} = \frac{1}{0.43} = 1.3$$

This proved that the hydrogen ion concentration was definitely the factor which inhibited the healing process in the previous investigation.

i.e. fourteen days, which proves conclusively that direct application of sodium ascorbate, in contradistinction to application of ascorbic acid, has no injurious effect on the healing of skin wounds. However, in rats, administration of Ascorbic acid by mouth, had no accelerating action on the healing of skin wounds.

2. THE RATE OF HEALING OF SECOND AND SUBSEQUENT WOUNDS.

Introduction.

In 1941 Young, Fisher and Young carried out a series of experiments on rabbits, the essential purpose of which was to discover whether or not the rate of healing of a second wound in the skin differs from that of a primary wound. A disc of skin was removed from the underlying aponeurosis on the animal's back in both initial and subsequent wounds, the latter being inflicted during the intermediate stages of healing of the previous generation of wounds - this was the one material respect in which they differed. In every one of ten groups, the subsequent wounds healed at a greater rate than the corresponding initial wounds and this increase in rate was statistically significant in seven groups. A conclusion was reached that some accelerating factor must operate in the healing of a subsequent wound which is lacking in the healing of an initial one.

This observation has been confirmed by Sandblom (1944) who measured the tensile strength of the healing wounds. It is conceivable that this acceleration may be due to a diffusible growth-promoting substance elaborated in and around the initial wounds. Frequently it has been postulated that damaged tissue liberates some substance capable of stimulating tissue proliferation - "growth-promoting substances" (Marchand,

1901,) "wound hormones" (Haberlandt, 1921). These substances, if they do exist, might reasonably be expected to be most abundant during the early and intermediate stages of healing when proliferative activity is most vigorous, i.e. from the tenth to the fourteenth day in experimental wounds of the type described above, and to be dissipated during the terminal stages of the process when proliferation is vanishing. No precise means are available of demonstrating these growth-promoting substances chemically or biologically but after Young, Fisher and Young's outstanding results it did seem most probably that their existence was justified.

Fischer (1930) has shown that tissue cultures which were repeatedly wounded mechanically, grew more quickly than controls, and that saline extracts of such wounded cultures could revive growth in cultures in which growth was latent. In 1941 Fischer concluded that "the liberation of substances with growth-promoting properties ..... which are set free by the mutilated cells" is one of the factors involved in the process of regeneration.

If such hypotheses are true and these "wound hormones" are liberated during the process of healing, the question arises as to whether they would still be present after complete healing and would then affect the rate of healing of a second wound inflicted shortly after the initial one had healed.

In all the work which has been accomplished on the rate of closure of "secondary" wounds, only the intermediate stages of healing of a previous generation of wounds has been investigated. If such substances as "wound hormones" exist during that stage in wound healing when proliferative activity is most vigorous, then it may well be expected for them still to be present after healing has been accomplished. With this speculation in mind, the following experiment was carried out on rats to discover an answer to this problem.

Method.

12 rats, which had already been wounded in the Ascorbic acid experiments, were used and second wounds were inflicted on the same site as the initial wounds, one month after the latter had healed. The same technical procedure for wounding as previously described was employed i.e. removal of discs of skin from the underlying aponeurosis on the outer aspect of each thigh. Both groups of rats received the same diet of rat cubes and water.

Results.

The wounds were closely observed from day to day to find the exact rate of healing. It was found that the average period of healing in the initial wounds was 16 days and in the second wounds 13 days.

The statistical significance of this can be seen in

Table 4. These results do suggest that initial wounds tend to have a lag in the final days of healing which is absent in subsequent wounds. Once again a possible explanation of this problem is the presence of "wound hormones." It would appear that these "chemical" substances remain during the entire period of healing and are still active some time after disappearance of the initial injury.

TABLE 4

The rate of healing of a second wound inflicted on the same area as the initial wound after one month.

12 rats with initial bilateral wounds - 24 wounds. Standard diet. Wounds healed in average of 16 days.

12 rats with second wounds - same animals. Standard diet. Wounds healed in average of 13 days.

No. of Wounds	Mean Period required for healing		Difference between Groups DAYS
	Days	± S.E. of mean	
24 I	16	± 0.33	3
24 S	13	± 0.45	

I = Initial wounds

S = Second wounds

$$\frac{m_I - m_S}{\sqrt{E_I^2 + E_S^2}} = \frac{3}{0.55} = 5.4$$

This Table shows that there is quite a significant statistical difference between the two groups.

SECTION B.

The Influence of Ascorbic Acid on Healing of Skin Wounds in Guinea Pigs.

Introduction

A) THIGH WOUNDS.

1. Oral Administration

Methods

Results

Histological Picture

2. Direct Application

Methods

Results

B) EAR WOUNDS.

1. Oral Administration

Methods

Results

THE INFLUENCE OF ASCORBIC ACID ON THE HEALING OF SKIN WOUNDS IN GUINEA PIGS.

Introduction.

The guinea pig, in contrast to the rat, is dependent on an outside supply of vitamin C to maintain proper metabolism in the body. Apart from the monkey it is the only laboratory animal in which scurvy can be experimentally introduced, and as a result intensive investigations have been carried out on this subject. The importance of this experimental work was recognised when it was found that the disease in animals bears a close relationship to that found in man. All results obtained in guinea pig experiments are not directly applicable to man especially in the selection of correct amounts of vitamin C required per day. Bourne concluded in 1942 that 2 mg. of ascorbic acid for a guinea pig may be approximately equal to 40 mg. for man. The results in Bourne's work suggest that 2 mg. is the optimum amount of vitamin C for securing regeneration of incised wounds in guinea pigs, and that dose levels higher than this will not improve the tensile strength of the wound. Although saturation with vitamin C is not essential for optimal healing, Bourne suggested that maximal saturation of tissues helps to provide best resistance of tissues to infection.

Danielli, Fell and Kodicek (1945) in their experiments on the effect of different degrees of vitamin C deficiency on

the differentiation of scar tissue, found that the optimum level of vitamin C for cavies, as assessed by its beneficial action on the rate of healing, is not less than 5 mg. per day and possibly even higher. This dose level varies considerably from that proposed by Bourne but this may have been due to a difference in ages of the animals used in the two experiments. Harris and Ray (1935) in their studies on vitamin C in the human being concluded that the vitamin C requirements of children are greater than those of an adult calculated per unit of body weight. This result may well apply to the guinea pig and before such conclusions as to the optimum dose required can be reached, careful study must be made to ensure that the animals are of the same age. With so much information available on the essential part ascorbic acid plays in the formation of intercellular material it is not necessary to stress the importance of taking a substantial dose of this vitamin daily.

Much important work has been accomplished in determining the effect of ascorbic acid deficiency on the scars of incised wounds - Bourne (1944); Hunt (1941); Wolbach (1926). Incisions in the abdominal wall and excisions of thigh muscle were the types of wounds inflicted on the animals and collagenous tissue thus played the most important part in the healing process. In the animals on a low dose there was an increased amount of reticulum in the scars which were far more

cellular and split up by extravasations of blood-stained fluid. This is indeed a direct contrast to the picture of a scar in a control animal - it is compact, uniform and vascular throughout, being composed of mature fibrocytes in a matrix of collagen.

The strength of a scar is dependent on the intercellular substance laid down by the fibroblasts and the full maturation of this substance, precollagen, to collagen - effective and rapid deposition is essential, but this process is itself dependent on the proliferation of mesodermal cells. Collagen arises as a condensation of the extracellular material in immediate association with the fibroblasts and their processes. In deficient animals the ready reversion of the collagen of scars to its immature and weaker form offers an explanation of the breaking down of healed wounds in scurvy.

Hunt found that cellular proliferation was little affected by deficiency during the early stages of healing. New fibroblasts appeared at first in both controls and deficients and multiplied to about the same extent. However, in the controls, this proliferation ceased about the fourteenth day after the operation but in scorbutic animals it continued long beyond that time. It may be the lack of ascorbic acid which stimulated proliferation of the cells or since the formation of scar tissue was delayed, the cellular proliferation was prolonged, but in all cases the cells remained immature.

The manner in which vitamin C acts in the formation of intercellular material is still unknown, despite the intensive research which has been carried out on this subject. So much attention has been concentrated on the formation and establishment of collagenous tissue in wound healing that the function of the other important tissue in this reparative process - epithelium, has been neglected. In view of this fact it thus seemed desirable to discover whether vitamin C deficiency affects in a similar manner the regeneration of epithelial tissue.

A) THIGH WOUNDS.

1. Oral Administration.

Methods.

Equal numbers of adult guinea pigs were used as Controls and Principals in all experiments. A basal diet of rat cubes, ascorbic acid-free, was given to the animals in both groups. To ensure that the Controls were fully saturated with the vitamin, 5 mgm. ascorbic acid were given to each animal every day. The Principals were each given ½ mgm. on alternate days thus reducing them to a subscorbutic state. The vitamin was freshly dissolved in water each day and the requisite dose was given by pipette in ½ c.c. water to each series. The weight of each guinea pig was noted every third day, and after the first two weeks on this diet, the

Principals' weights began to decrease steadily while the Controls regularly increased. - Fig. 7. The Principals by this time were beginning to show the characteristic signs and symptoms of deficiency. Tender, inflamed limbs were frequently observed, but most noticeable of all was the rough, dull hair. The animals often developed the sign of "staring fur," in which the hair on chest and abdomen became wet and matted - a sign frequently correlated with a nutritional defect. There was no visible disturbance of the gum in any case and pressure did not produce any bleeding or other symptom often associated with vitamin C deficiency in man.

The animals were kept on this special diet for approximately three weeks before the operation. 30 guinea pigs were used in this experiment and the wounds were made under ether anaesthesia using the same method described for the rat. A cork borer of larger diameter, approximately 18 mm., was used this time to demarcate the area on the thigh - the hair having previously been clipped - since the surface area of the guinea pig is of greater dimension than the rat.

Sections of the disc of skin were made showing the epithelial layer which had been removed. (Fig. 8). A serial picture of the healing process was prepared histologically in both groups and the results of the wide contrast observed in the experiment can be clearly seen. (Figs. 9 - 14.)

FIGURE 7.

Graph showing the weight curves of a Control, receiving excess Ascorbic acid, and a Principal, on a sub-scorbutic diet.

Control:- Standard diet, 5 mg. Ascorbic acid daily by mouth in solution.

Principal:- Standard diet, ½ mg. Ascorbic acid on alternate days by mouth in solution.

Striking difference in graphs of two animals. Control's weight increases normally, while Principal's weight rises for a short time and then rapidly decreases especially after the operation.

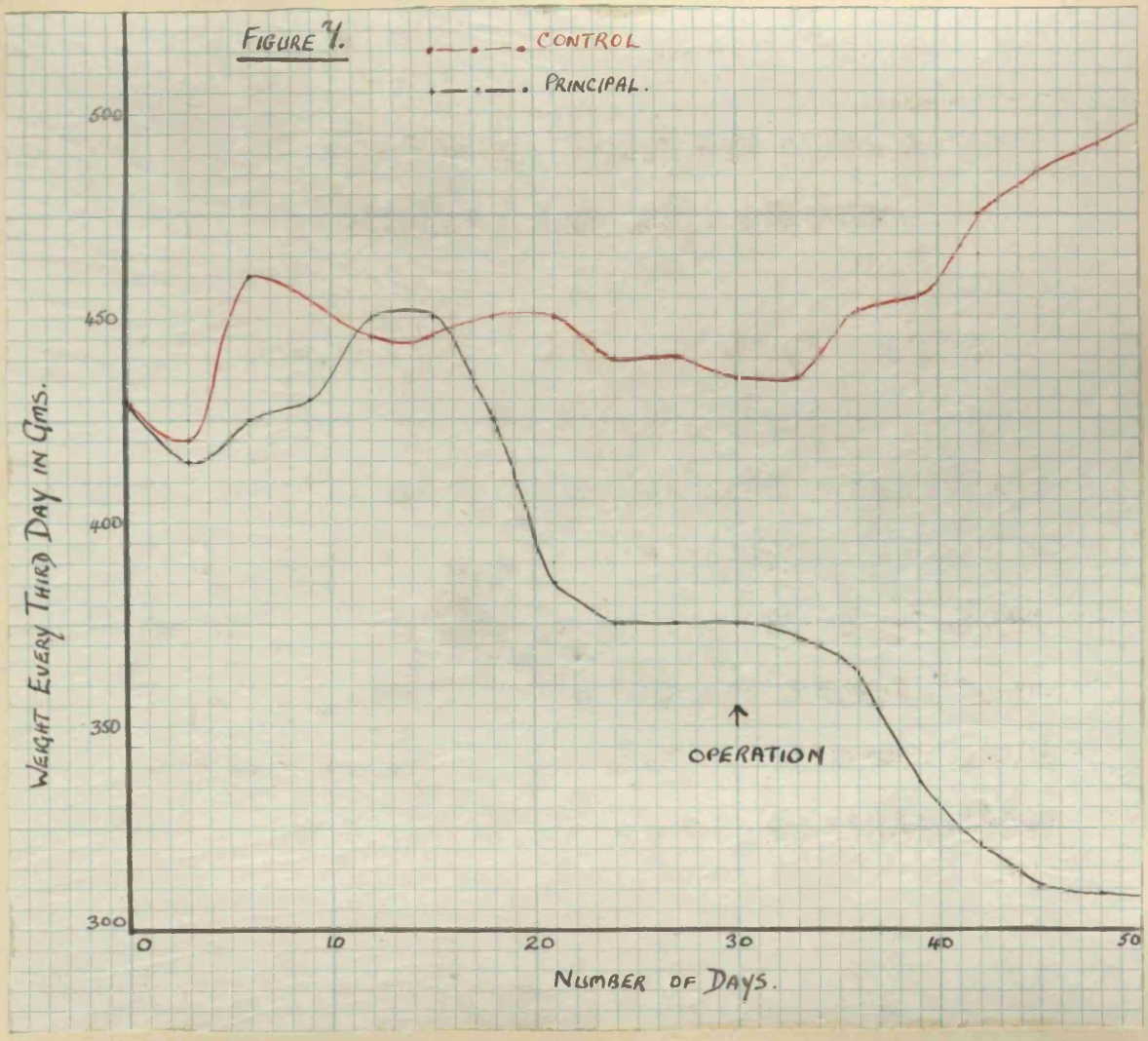
FIGURE 4.
CONTROL
PRINCIPAL.
500
450
400
350
300
WEIGHT EVERY THIRD DAY IN GMS.
OPERATION
0
10
20
30
40
50
NUMBER OF DAYS.

FIGURE 7

Results.

A striking difference from the results obtained previously with the rats became obvious when healing commenced. At first scab formation took place in the wounds of both groups, but in the Principals it was soon obvious that this was very superficial as haemorrhages occurred daily and at the slightest pressure the scabs loosened and came off. Epithelialisation was greatly delayed, thus leading to a significant difference between the periods of healing. Figs. 11 and 12 show pictures of the wounds taken on the 16th day after the operation and the pronounced delay in healing of the Principals is quite clearly shown here. There is only a minute scab left on the Control while the wound in the Principal is still of large area and shows little sign of healing. There was a slight scatter in the time of healing in each group but the average periods of healing were:-

Controls ..... 16 days.

Principals ..... 29 days.

TABLE 5

Effect of oral administration of Ascorbic acid on healing of skin wounds.

Control:- Standard diet, 5 mg. Ascorbic acid daily by mouth in solution. 12 animals with bilateral wounds - 24 wounds which healed in average 16 days.

Principal:- Standard diet, ½ mg. Ascorbic acid on alternate days by mouth in solution. 12 animals with bilateral wounds - 24 wounds which healed in average 29 days.

No. of Wounds	Mean Period required for healing		Difference between Controls and Principals DAYS
	Days	± S.E. of mean	
24 C	16	± 0.81	13
24 P	29	± 1.15	

$$\frac{m_p - m_c}{\sqrt{E_p^2 + E_c^2}} = \frac{13}{1.14} = 9.2$$

The statistical difference of this delay, as determined by Burn's formula, proves conclusively that it is most significant.

FIG. 8

Fig. 8 shows a picture of the layer of skin actually removed at the operation. Both epidermis and cutis vera were cut away but no underlying muscular tissue.

(X 90)

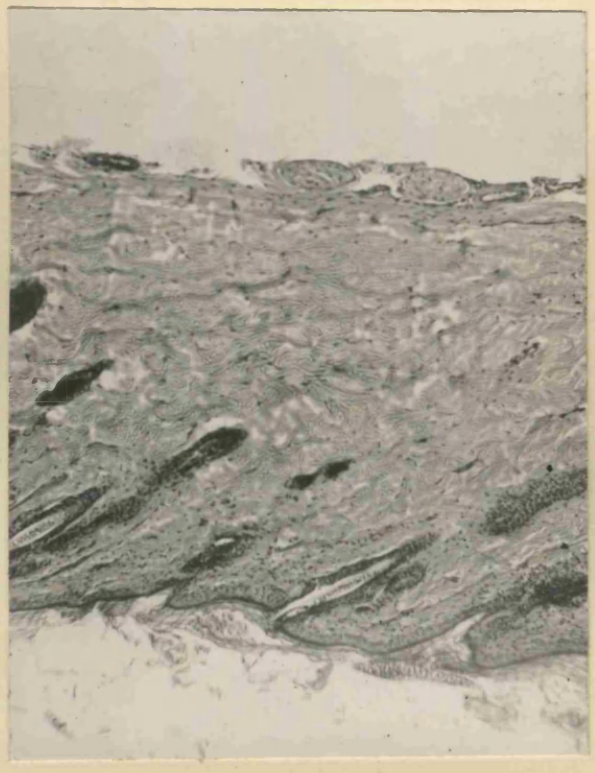

FIGURE 8

Figs. 9 and 10.

Fig. 9 - 2nd day Wound

Control:- Epithelium already spreading in from the edge of damaged surface. Small well-formed scab covers the whole wound. Fine, wavy collagen fibres can be seen between the cells and fibroblasts and are already beginning to invade wound in an orderly orientation.

(X 90)

Fig. 10 - 2nd day Wound

Principal:- No sign of new epithelial growth. A mass of haemorrhagic, oedematous tissue densely infiltrated with polymorphs fills the cavity which is covered by a large, thick scab. No collagen fibres can be seen and the fibroblasts are few and orientations chaotic.

(X 90)

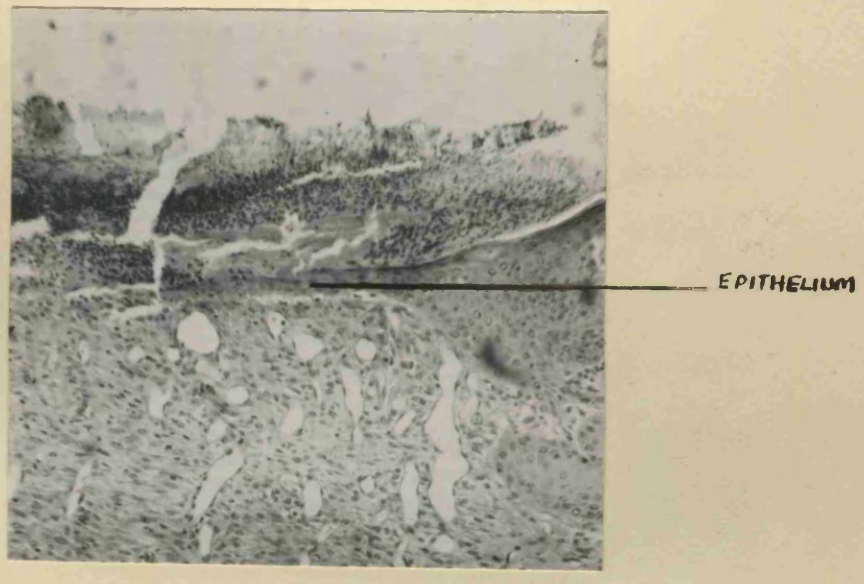

FIGURE 9

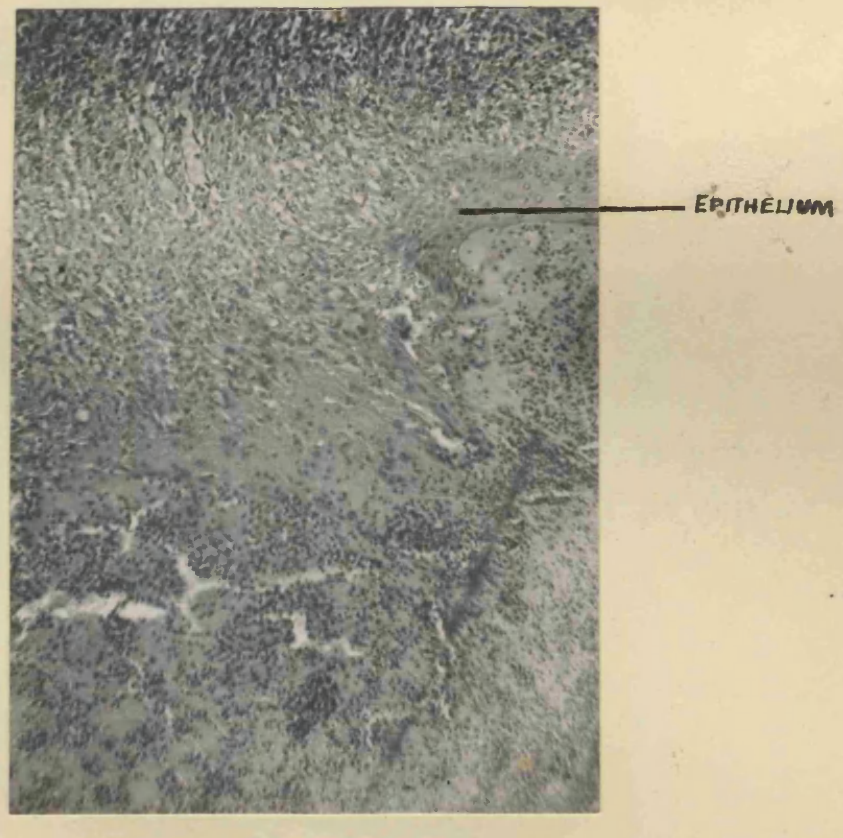

FIGURE 10

Figs. 11 and 12.

Fig. 11 - 16th day Wound

Control:- Complete growth of epithelium over healed area. In the dermis parallel layers of thick collagen fibres and fibroblasts can be seen. No new formation of hair follicles or sebaceous glands has developed.

(X 90)

Fig. 12 - 16th day Wound

Principal:- Wound still covered by thick scab, the histological character of the scar remaining unchanged. Orientation of both cells and fibres is still chaotic and there is little outgrowth from the epithelium.

(X 90)

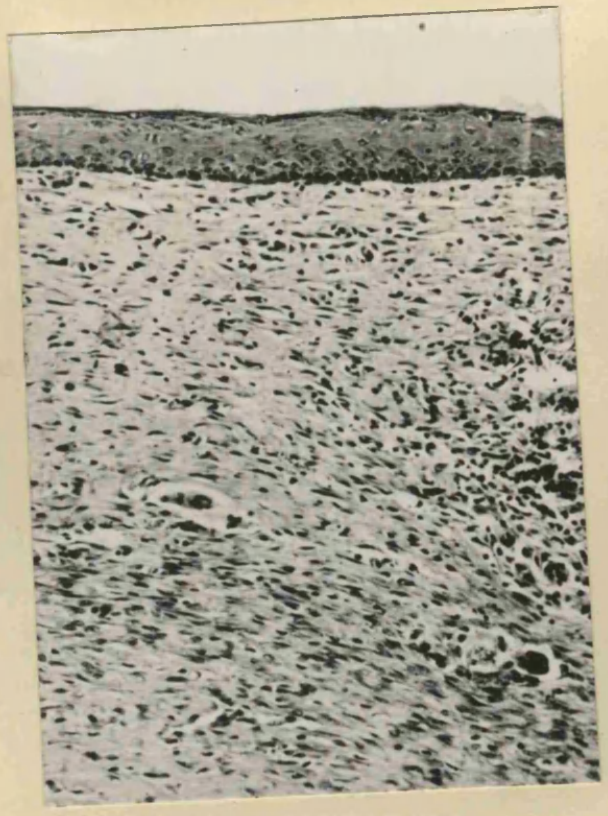

FIGURE 11

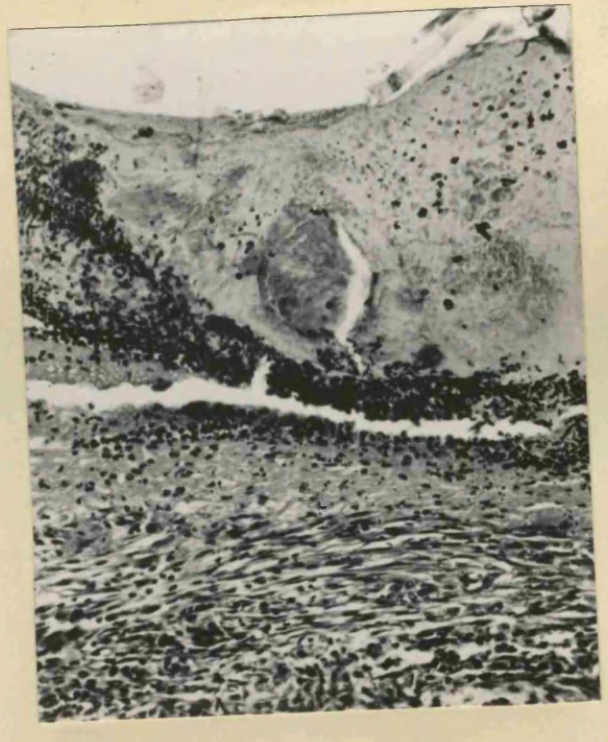

FIGURE 12

Figs. 13 and 14.

Fig. 13 - 30th day Wound.

Control:- Histological picture of epithelium and dermis back to normal. New sebaceous glands and hair follicles forming. Well-formed fibroblasts and collagenous tissue. (X 90)

Fig. 14 - 30th day Wound.

Principal:- Epithelialisation now complete. No dermal papillae projecting upward into epidermis, and no sign of any hair follicles or glands. In dermis fibroblasts have still no proper formation and collagen fibres are very scanty. (X 90)

FIGURE 13

FIGURE 14

Histological Picture.

In order to obtain a histological picture of the reparative process in each group, 3 Controls and 3 Principals were killed at intervals - on 2nd day after operation, 16th day (Control healed) and finally on the 30th day when the Principal had healed. This microscopical picture shows in detail the delay in healing - especially the lack of epithelialisation in the Principal's wound on the 16th day when the Control was healed.

From the above results it may well be deduced that, when an area of skin is removed in an animal liable to suffer from ascorbic acid deficiency, administration of ascorbic acid is indicated.

2) DIRECT APPLICATION

Since the result of general ascorbic acid deficiency has such a significant retardation on the healing of skin wounds, it seemed worth while therefore to try the effect of direct application of ascorbic acid to the thigh wounds in Controls and Principals.

Methods.

The animals were divided into four groups:- a) 4 Controls receiving 5 mgm. ascorbic acid orally per day, with Ringer applied to both wounds (pH Ringer approx. 7.4); b) 4 Controls receiving the same oral dose but both wounds painted daily with 3% sodium ascorbate (pH regulated to approximately

6.6, since ascorbic acid is so quickly destroyed at alkaline pH); c) 4 Principals receiving ½ mgm. of ascorbic acid on alternate days orally, both wounds being treated with Ringer; d) 4 Principals with same oral dose and both wounds painted with 3% sodium ascorbate daily. Ringer was used in groups (a) and (c) in order to keep the wounds moist, so that the only difference in solutions would be the ascorbic acid content. The animals were wounded under ether anaesthesia on the thigh in the usual manner and all wounds painted daily with the requisite solution.

Results.

It was found that in the two Control groups, both receiving ample vitamin C by mouth, there was little or no difference in the rate of healing. The ascorbic acid appeared to accelerate the healing at first, but this proved to be of no significance as seen from Table 6. The average periods of healing were:-

Control Wounds painted with ascorbic acid.....15 days.
Control Wounds painted with Ringer.....16 days.

This result seems to correlate with that obtained in the rat experiments on page 37 - "buffered" ascorbic acid in excess appears to have a negligible effect on the rate of healing of skin wounds, if the supply of ascorbic acid is otherwise normal.

However a different picture was obtained in the case of the Principals' wounds. Here there was a notable acceleration

TABLE 6

Effect of direct application of Ascorbic acid solution on Control Guinea pigs.

Two groups, each with 4 animals.

a) 4 Controls receiving 5 mg. Ascorbic acid orally by mouth, with Ringer applied to both wounds. Bilateral wounds.

b) 4 Controls receiving the same oral dose but both wounds painted daily with 3% sodium ascorbate. Bilateral wounds.

a) 8 wounds healed in 16 days.

b) 8 wounds healed in 15 days.

No. of Wounds	Mean Period required for healing		Difference between Groups
	Days	± S.E. of mean	DAYS
8 CR	16	± 0.66	1
8 CA	15	± 0.46	

CR = Control with Ringer
CA = Control with Ascorbic acid

$$\frac{m_{CR} - m_{CA}}{\sqrt{E_{CR}^2 + E_{CA}^2}} = \frac{1}{0.81} = 1.23$$

This result proves to be of no significance and it appears that excess Ascorbic acid in Guinea pigs has no accelerating effect on healing of skin wounds.

in the healing time and an average difference of 10 days was observed between the two groups. The outward appearance of the wound treated with ascorbic acid began to assume a more normal appearance as healing progressed. The injured area did not become so inflamed, nor did the scab tend to break open at regular intervals as was the case in the Principals' wounds treated with Ringer. Group (d) did not heal so rapidly as the Control groups of this and the previous series but on the average all wounds had healed by the 20th day after operation. - Table 7. In contrast, group (c) had an average period of healing of 30 days c.f. 29 days in series shown on p.50. There is no doubt that direct application of sodium ascorbate to a skin wound in a vitamin C deficient cavie accelerates healing.

In consequence of this important observation, a further experiment was carried out on a group of Principals to determine whether the action of ascorbic acid on the healing process was local or systemic.

Ten Principals, receiving ½ mgm. ascorbic acid orally on alternate days, were wounded on both thighs. As far as their diets were concerned, then, all were on a scorbutic diet. Each wound on the right thigh was painted daily with the ascorbate solution, and the left wound painted daily with Ringer. At first it appeared as if the right wound would heal more quickly but in the intermediate stages of healing

TABLE 7

Effect of direct application of Ascorbic acid solution on Deficient Guinea pigs.

Two groups, each with 4 animals.

a) 4 Principals receiving ½ mg. Ascorbic acid orally on alternate days, both wounds being treated with Ringer. Bilateral wounds - 8 wounds which healed in 30 days.

d) 4 Principals receiving the same oral dose and both wounds painted with 3% sodium ascorbate daily. Bilateral wounds - 8 wounds which healed in 20 days.

No. of Wounds	Mean Period required for healing		Difference between Groups
	Days	± S.E. of mean	DAYS
8 PR	30	± 0.66	10
8 PA	20	± 2.16	

PR = Principal with Ringer
PA = Principal with Ascorbic acid

$$\frac{m_{PR} - m_{PA}}{\sqrt{E_{PR}^2 + E_{PA}^2}} = \frac{10}{2.24} = 4.4$$

This result shows that there is a significant delay in healing and there is no doubt that direct application of Ascorbic acid to a skin wound in a vitamin C deficient Guinea pig accelerates the healing time.

the wound on the left thigh caught up with the wound on the right thigh. Ultimately the period of healing of the right wound was 23 days and that of the left 24 days. This difference is not significant, Table 8. The obvious suggestion is that the ascorbic acid is absorbed from the raw surface and exerts its action systemically. These two periods of healing lie mid-way between the healing times of groups (c) and (d) in the previous experiment. This result is more or less to be expected if it is accepted that Ascorbic acid acts systemically, as there is only half the amount of solution painted on the wounds as there was in group (d) of the previous series. There is one possible fallacy to this latter series, the cavies may have licked their wounds. It was not found practicable to take certain steps to avoid this danger, but at no time was licking observed.

B) EAR WOUNDS

1) Oral Administration

After the conclusive observations that Ascorbic acid deficiency has a direct effect on the healing of skin loosely attached to deeper structures, it seemed desirable to discover whether the same result could be obtained in the ear where the skin is rigidly fixed. In actual fact the ear wound is essentially similar to the thigh wound as it is mainly epidermis which is

TABLE 8

Effect of Ascorbic acid painted on right wound of Principal while left wound is treated with Ringer.

Ten animals on standard diet, each receiving ½ mg. Ascorbic acid on alternate days. Each wound on right thigh painted daily with 3% sodium ascorbate, and left wound treated daily with Ringer.

No. of Wounds	Mean Period Required for healing		Difference between Right and Left wounds DAYS
	Days	± S.E. of mean	
10 R	23	± 1	1
10 L	24	± 1.6	

R = Right wound
L = Left wound

$$\frac{m_L - m_R}{\sqrt{E_L^2 + E_R^2}} = \frac{1}{1.9} = 0.53$$

The difference in healing time between right and left wounds is not significant which proves that Ascorbic acid does not have a local action but is absorbed from the surface and exerts its action systemically.

affected. Only the surface area of the epithelium removed is very much reduced and since the epithelium would be able to proliferate quickly on both sides of the ear, it was expected that an acceleration in the reparative process would be observed.

Methods.

The animals were kept on the usual diet of rat cubes and water, with the Controls receiving 5 mgm. ascorbic acid orally per day and the Principals ½ mgm. on alternate days, for two weeks before the operation. General anaesthesia was administered using ether. A marginal strip, roughly 1 cm. long, was cut off the tips of both ears in 40 Controls and Principals.

Results.

Proliferation of epithelium took place quickly, being particularly noticeable in the Controls, as on the first day after the operation the tips of the ears were rounded and healing well. At the operation it was noted that the Principals tended to bleed more profusely than the Controls which was to be expected because of the increased capillary fragility. Healthy epithelium soon grew quickly over the tips of the Controls' ears and in an average of 8 days all wounds had healed. There was a noticeable delay in the repair of the Principals' wounds which proved to be significant - Table 9.

TABLE 9

Effect of oral administration of Ascorbic acid on healing of ear wounds.

Control:- Standard diet, 5 mg. Ascorbic acid daily by mouth in solution. 20 animals wounded in each ear - 40 wounds which healed in 8 days.

Principal:- Standard diet, ½ mg. Ascorbic acid on alternate days by mouth in solution. 20 animals wounded in each ear - 40 wounds which healed ih 14 days.

No. of Wounds	Mean Period required for healing		Difference between Controls and Principals DAYS
	Days	± S.E. of mean	
40 C	8	± 0.6	6
40 P	14	± 0.7	

$$\frac{m_P - m_C}{\sqrt{E_P^2 + E_C^2}} = \frac{6}{0.925} = 6.4$$

This result is of statistical significance.

A thick scab remained on the Principals' ears for some time and haemorrhages occurred at the slightest pressure. The ears were very sensitive to the touch and appeared to be inflamed to some degree. Complete epithelialisation did not take place until an average of 14 days after the operation. This result certainly agrees with the previous experiment on thigh wounds and stresses the importance of ascorbic acid in the healing of skin epithelium.

SECTION C

The Influence of Ascorbic Acid on the Healing of Corneal Epithelium in Guinea Pigs.

Introduction

1. Oral Administration

   Methods

   Results

THE INFLUENCE OF ASCORBIC ACID ON THE HEALING OF CORNEAL EPITHELIUM IN GUINEA PIGS.

INTRODUCTION.

It is well known that the epithelium of the cornea is endowed with a remarkable capacity for regeneration and the repair of wounds may be accomplished in a matter of hours. This is surprising when it is realised that the cornea is avascular. Neither blood vessles nor lymphatics occur in the propria. They are halted at the limbus, where the cornea merges with the sclera. This is in sharp contrast to the rich supply of both immediately beneath the epidermis of the skin. The epithelial cells of the cornea are indeed several millimetres distant from the nearest capillaries. Consequently the abundant nerve supply of the cornea has nothing to do with blood vessels, nor with any kind of muscle. It is sensory. Yet, in the absence of definite channels for supply of materials of haematogenous origin, and depending as it does on slow diffusion, the corneal epithelium is extraordinarily adept at repair of injuries, (Cowdry, 1938).

The method of regeneration of corneal epithelium still remains a point of controversy in Ophthalmological literature. Ranvier (1889) stated that the process of filling up an epithelial defect is simply mechanical, release of tension allowing the cells to slide over Bowman's membrane and spill

over into the bare area. This conclusion was based on the observation that mitosis did not usually become apparent for about 12 hours after injury. Weinstein (1903) and Matsumoto (1918) both agreed to some extent with this statement although 1 hour after the operation the former found mitosis in the basal cells near the wound, but not at edge, and over the entire cornea in 4 hours. Arey and Covode (1937) have followed the proliferative activity in corneal wounds and found that during the period of epithelial repair mitotic frequency fell off to less than one-half the normal amount. Although wound closure was complete in six to ten hours it was not until after the fourth day that the mitotic rate returned to normal. During the fifth day there was a marked acceleration to exceed the normal rate by 75% followed by a return to normal. From other literature on the subject - Hartwell (1929); Parsons (1904) it seems to be quite obvious that both processes undoubtedly occur i.e. ingrowth of epithelial cells from the sides and later karyokinesis, but the problem still remains as to the actual time when mitosis begins.

The relationship between Vitamin C and the cornea has not yet been definitely settled. Vitamin C is known to exist in high concentrations in the human cornea as well as

the lens, its probable function being to maintain balance between the processes of reduction and oxidation. On the assumption that it is an important factor in the nutrition of the structure of the eye, Lyle and McLean (1941) used it in cases of inflammatory conditions of the cornea. Doses of 500 mgm. of Vitamin C were given once a day intravenously, as well as ordinary local treatment, until active inflammation of the eye had ceased, after which the vitamin was given orally. They stated that an "improvement in most cases is almost dramatic." There was no reason to believe that a Vitamin C deficiency existed, and the beneficial results were attributed to flooding the body with excess Vitamin C.

Livingstone and Walker (1940) state that direct mustard gas contamination of the eye in rabbits can be considerably mitigated by high doses of Vitamin C. Although Mann and Pullinger (1940) repeated this experiment, they could not confirm that injections of Vitamin C influence the progress of mustard gas lesions of the eye in rabbits. These mustard gas lesions must undoubtedly affect more than the outer epithelium, due to the severe local action of the gas and cases of ulceration are bound to occur. The healing of these inflammatory lesions are bound to be dependent on the healing of the substantia propria as well as the epithelium and so do not give a true picture of healing of the corneal epithelium.

A new method was thus devised to introduce a surface injury which would only destroy the outer layers of epithelium, so that the regeneration could be followed in both Controls and Principals.

1. ORAL ADMINISTRATION

Method

The same basal diet, rat cubes and water, was given to 50 Controls and Principals as previously with the usual oral dose of ascorbic acid. The animals were kept on this diet until the Principals began to show deficient symptoms - approximately two weeks - and then wounded.

A "standard wound" is required when the rate of healing is to be compared in two groups. Several methods for abrading the cornea have been described before, but the accuracy of the wounds is open to question. Gundersen and Liebman (1944), in their experiment on the effects of local anaesthetics on regeneration of corneal epithelium, made abrasions 2mm. wide with a metal spatula, from limbus to limbus, across the centres of the cornea. Schaeffer (1946), studying the effects of amino acids on wound healing, made wounds using a trephine to mark the area on the cornea and abrading the epithelium with a spatula. Post (1923) used a thermophore applied at 130° for 1 min. to the cornea, but this heating often caused more of an injury than the average trauma affecting exfoliation of the corneal epithelium.

However these methods involve a long and tiresome operation in which the size and depth of the wound often varies to some degree. The following method was therefore introduced to reduce the time of operation to a minimum and to ensure that each lesion was of similar area.

General anaesthesia was administered using ether and in addition a local anaesthetic, Holocaine, was placed in the conjunctival sacs. It is quite safe to use this drug locally as Post (1923) observed that Holocaine has no material effect on the rate of replacement of epithelium in the cornea. Before the injury was inflicted, a drop of fluorescein was placed in each eye and after a few minutes the excess was washed off with Ringer. The degree of wounding could thus be observed immediately as the circular abrasion appeared bright green whenever the instrument came in contact with the cornea. The operation was carried out in daylight, but the intensity of fluorescence was enhanced when an ultra-violet lamp was also present.

A dental fissure burr of 2 mm. diameter, attached to a dental engine supplying the motor power, was used to abrade the corneal epithelium in both eyes. This burr has a cutting face, and the face only was used. The cutting face was applied with a firm, steady pressure. The cornea gives

before the pressure and there seems to be no fear of penetrating the substance of the cornea. The wound was of uniform depth and only corneal epithelium was removed - see Fig. 15. Unfortunately, due to the rotary motion of the cutting face a spot of epithelium was very apt to remain in the centre of the abrasion. This central spot could be removed, however, by running round the periphery of the abrasion with a burr of smaller diameter. The area chosen for the abrasion was as close to the centre of the cornea as possible so that the subsequent observations could be easily made.

Examinations were made every few hours because of the short healing time. Each time a drop of fluorescein was placed in each eye and the excess fluid washed off with Ringer as before. The injured area was then examined under U.V. light until complete epithelialisation of the cornea took place. Corneal spectacles were worn during the observations so that the exact end point of healing could be noted.

Results

Healing proved to be most rapid and it was essential to follow the progress of healing at least thrice daily. The relative divergencies in the end point of healing were much wider than in any previous experiment with skin wounds, complete epithelialisation being noted at 23 hours in one case while

others took as long as 72 - 90 hours. However, it was found that the average periods of healing in Controls and Principals were approximately the same, the difference being 1 hour. From Table 10 it is seen that the result was not statistically significant.

Histological Picture. In order to follow the process of healing microscopically, one guinea pig was killed a) immediately after operation, b) 20 hours after operation, c) when cornea was practically healed - 40 hours. At 20 hours (Fig. 16) it can be seen that the epithelial cells are sliding over the denuded area but there is no sign of mitosis in the epithelium. Immediately beneath the epithelial layer in the substantia propria however, there can be seen a dense congregation of wandering cells which are really emigrant leucocytes. These lie around the corneal cells and are only to be found massed together like this at the wounded area. By 40 hours (Fig. 17) all signs of this collection of leucocytes have dispersed and the connective tissue once again looks normal. The denuded area is now completely covered with epithelium, and apart from one small spot in the centre on the extreme exterior side, the cornea appears to be perfectly normal. This picture quite clearly demonstrates how efficient the cornea is at repair of superficial injuries and no defect ever remains.

TABLE 10

Effect of oral administration of Ascorbic on healing of corneal epithelium.

Control:- Standard diet, 5 mg. Ascorbic acid by mouth daily. 25 animals wounded in each eye - 50 wounds which healed in 45 hours.

Principals:- Standard diet, ½ mg. Ascorbic acid by mouth on alternate days. 25 animals wounded in each eye - 50 wounds which healed in 46 hours.

No. of Wounds	Mean Period required for healing		Difference between Controls and Principals HOURS
	Hours	± S.E. of mean	
50 C	45	± 9	1
50 P	46	± 10.2	

$$\frac{m_p - m_c}{\sqrt{E_p^2 + E_c^2}} = \frac{1}{13.6} = 0.073$$

This result has no statistical significance.

Conclusion. This result proved to be rather surprising when it is known that the % of vitamin C present in the cornea is high. The absence of any influence on the wounds does seem to suggest that ascorbic acid deficiency only affects the healing of wounds when connective tissue is present and that the power of epithelial proliferation is not impaired.

Fig. 15

Fig. 15 gives a picture of the wounded area on the cornea immediately after the operation. It can be seen that the wound is of uniform depth and only the epithelium is removed. The substantia propria is not injured in any way. (X 90)

FIGURE 15

Figs. 16 and 17

Fig 16 shows the injured corneal epithelium 20 hours after the operation. No sign of mitosis in the epithelial layers but there is a dense congregation of leucocytes in the substantia propria immediately beneath the wounded surface. (X 90)

Fig. 17 shows the corneal epithelium has practically healed apart from a slight defect in the centre. Apart from this the cornea has healed so well in the short time of 40 hours that there is little sign of the operation which had been performed only a short time before. (X 90)

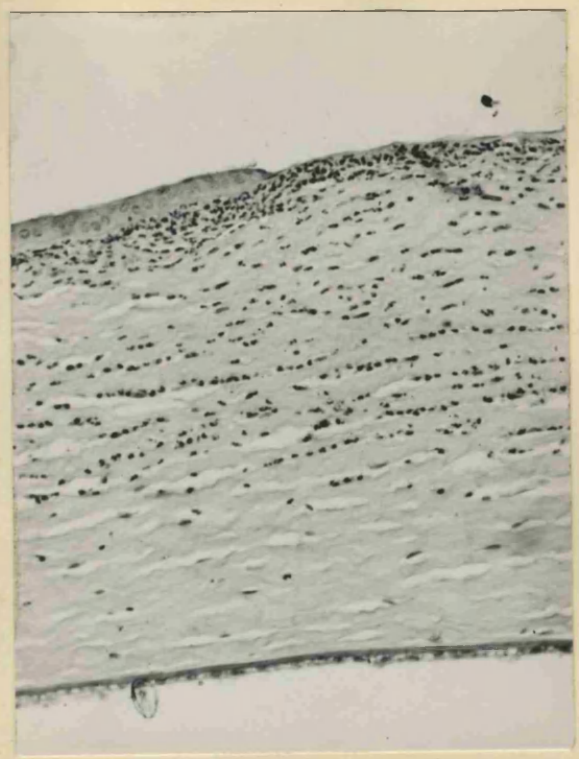

FIGURE 16

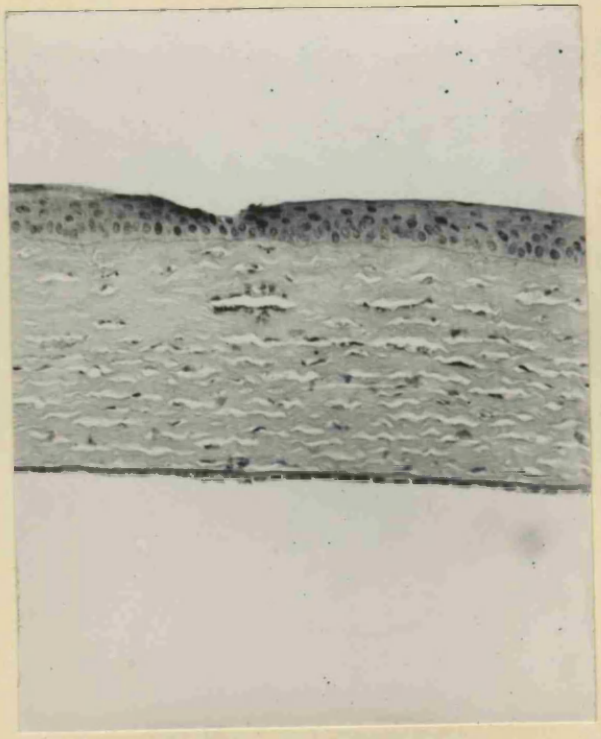

FIGURE 17

SECTION D.

The Influence of Ascorbic Acid on Healing of Muco-periosteum of Gum in Guinea Pigs.

Introduction

1. Oral Administration of Ascorbic Acid.

   Methods

   Results

   Discussion

2. Oral Administration of Orange Juice.

   Methods

   Results

The Influence of Ascorbic Acid on Healing of Muco-periosteum of Gum.

Introduction.

The intercellular substances also regulated by vitamin C include those of dentine, the matrices of bone and possibly of the capillary walls. Vitamin C, therefore, plays a part in the nutrition of the teeth.

As early as 1919 Zilva and Wells showed that radical changes occur in the teeth of scorbutic animals. Failure of odontoblasts and osteoblasts to form the collagenous matrices of dentine and bone were symptoms of severe deficiency. In the continuously growing incisors and molars of the guinea pig, the periodontal tissues are replaced rapidly, so that the scorbutic effects are usually not so outstanding as in man. However the gross, X-ray and histologic variations in the periodontal tissues of the guinea pig maintained on diets deficient in Ascorbic Acid are pathognomonic of scurvy and reproduce the diagnostic features recognised as characteristic of human diffuse atrophy pyorrhea, (Boyle, Bessey and Wolbach, 1937).

The incisor teeth of a guinea pig are a more delicate criterion of the presence of scurvy than bones. Within 4 to 5 days the odontoblasts may be seen to shorten and become separated from the dentine by a faintly staining fluid zone - Eddy and Dalldorf (1941). Fish and Harris (1935) also

found in the teeth of scorbutic guinea pigs, enamel defects which they believed to be related to caries. Boyle (1938) has described the effects of scurvy on the alveolar bone in guinea pigs and states that vitamin C deficiency is the only nutritional disease, in his experience, which produces the characteristic features of systemic pyorrhea. After several months of a partial vitamin C deficient diet the animals showed periodontal weakness and many teeth became displaced. However satisfactory evidence of the relation between Ascorbic Acid deficiency and dental caries has still not been established. - Westin (1925); Grandison, Stott and Cruickshank (1942).

Atrophic changes in the enamel epithelium appear considerably later than do changes in the odontoblasts - Boyle (1938). The lack of collagen-fibre formation in the periodontal membrane weakens that structure on the lingual surface of the tooth and leads to failure of incisal stresses, so that injury to the enamel epithelium on the labial surface results. Fish and Harris (1935) believe that the atrophy of the enamel epithelium is caused directly by vitamin C deficiency.

The importance of vitamin C in the treatment of gingivitis has been reported by many with favourable results - Campbell and Cook (1941); Roff and Glazebrook (1940);

Kent (1943); Stuhl (1943). The use of vitamin C in gingivitis is based on the assumption that this process is either due to a scorbutic condition or that it is improved by the administration of extra ascorbic acid. Numerous investigators have also combined vitamin C therapy with dental hygiene and dental procedures and have reasoned that such a form of treatment gave satisfactory results.

Campbell and Cook (1942) observed in patients at a clinic that the oral administration of vitamin C before and after dental extractions plays an important role in the healing of wounded gum tissue and the absorption of the alveolar bone margines. In addition, they observed less postoperative pain and bleeding. This observation has not been further investigated, apart from the clinic, and it was with the intention of studying the process of healing of gum tissue that the following work was initiated.

It is quite obvious from the above discussion that vitamin C is essential in the development of the tooth, but little or no mention is made of its effect on periosteum. The surface epithelium of the muco-periosteum is typically stratified and squamous with a covering keratinous layer which is essentially similar to the epithelium previously studied. The actual wound made in the experiment injures more than the surface epithelium as no satisfactory method is available by which only this layer can be removed.

However, as it was the effect of ascorbic acid on wounded gum tissue, which was to be studied, this method proved to be most useful.

1. ORAL ADMINISTRATION

Methods.

The guinea pigs were placed on the usual diet of rat cubes and water, supplemented with the respective doses of ascorbic acid. Thirty Principals and Controls were kept on the diet for three weeks before the operation.

The following method was devised for making experimental wounds in the gum. The two most suitable sites in the mouth chosen for wounding were the diastema of the upper jaw and the front of the lower jaw, as they enabled one to observe the healing process quite easily. A specially made trephine for a dental engine was used to cut a small cylinder from the muco-periosteum of the gum right down to the bone. The size of the wound had perforce to be small being 3 mm. in diameter. Forceps were used to pluck out the cylinder of muco-periosteum. Only slight bleeding occurred at the operation and sepsis was observed in none of the wounds.

Results.

This wound, in contrast to the corneal injury, removed not only the layers of stratified epithelium but also the submucous tissue of the muco-periosteum - in fact it was very similar

to the skin wound. A most unexpected result was obtained, however, which is difficult to explain. The rate of healing of such gum wounds was similar in the Controls and Principals, the average period for complete epithelialisation being 6 days. No statistical significance was found, as is seen from Table 11.

Discussion. These negative results from gum wounds are difficult to explain. New formation of collagen should have played a part in their repair. Although the epithelium may have grown across in spite of defective collagen formation it did not appear to be so, as there was no tendency of the wounds to break open after healing had taken place. It was most surprising at times to find the Principals healing slightly quicker and better than the Controls.

One possible explanation, which was followed up (vide infra,) comes from reports by Todhunter and co-workers (1940) and Scarborough (1938, 1939, 1940) who observed that extracts from orange juice produced better effects in maintenance of capillary resistance, in man and guinea pig, than pure synthetic vitamin C. At the moment evidence for the clinical value of vitamin P in the absence of a deficiency of the vitamin is scanty, but it seemed most desirable to find out if the cause of the negative results in the healing of the gum wounds was due to the absence of another dietary

TABLE II

Effect of oral administration of Ascorbic acid on healing of Muco-periosteum of the gum.

Controls:- Standard diet, 5 mg. Ascorbic acid daily by mouth in solution. 15 animals with two wounds in upper jaw - 30 wounds which healed in 6.2 days.

Principals:- Standard diet, ½ mg. Ascorbic acid by mouth on alternate days. 15 animals with two wounds in upper jaw - 30 wounds which healed in 6 days.

No. of Wounds	Mean Period required for healing		Difference between Controls and Principals DAYS
	Days	± S.E. of mean	
30 C	6.2	± 0.32	0.2
30 P	6	± 0.8	

$$\frac{m_c - m_p}{\sqrt{E_c^2 + E_p^2}} = \frac{0.2}{0.86} = 0.24$$

The difference between the two groups is not statistically significant.

factor.

2. ORAL ADMINISTRATION OF ORANGE JUICE.

Methods.

Ten Controls and 10 Principals were given the usual diet of rat cubes and water. The Principals received the customary dose of ½ mgm. of ascorbic acid on alternate days. The Controls this time were given concentrated orange juice, the dose containing the equivalent amount of ascorbic acid as before - 5 mgm. every day. The animals were kept on this diet for the usual three weeks before wounding. Two wounds were made in the upper jaw in each animal as in the previous experiment (vide supra) with the dental trephine.

Results.

No change in the previous results was observed. Once more the wounds healed in an average of four to five days, the Controls again taking slightly longer than the Principals but with no significant difference - Table 12. It appears therefore that the pure synthetic ascorbic acid is just as efficient in the healing process as the orange juice and that the presence of another factor, such as vitamin P, plays no part in the explanation of the Controls' slight retardation.

This result seems to confirm that although vitamin C is of great importance in the maintenance of the teeth, it plays an insignificant part in the repair of wounded gum tissue.

TABLE 12

Effect of oral administration of Ascorbic acid on healing of Muco-periosteum.

Control:- Standard diet, with orange juice daily. 10 animals, with two wounds in the upper jaw - 20 wounds. Wounds healed in 5.2 days.

Principal:- Standard diet, with ½ mg. Ascorbic acid on alternate days. 10 animals, with two wounds in the upper jaw - 20 wounds which healed in 4.3 days.

No. of Wounds	Mean Period required for healing		Difference between Controls and Principals DAYS
	Days	± S.E. of mean	
20 C	5.2	± 0.64	0.9
20 P	4.3	± 0.96	

$$\frac{m_c - m_p}{\sqrt{E_c^2 + E_p^2}} = \frac{0.9}{1.53} = 0.53$$

The difference in healing times between Controls and Principals has no statistical significance.

PANTOTHENIC ACID

SECTION A

SECTION A

THE INFLUENCE OF PANTOTHENIC ACID ON HEALING OF SKIN WOUNDS IN RATS.

(A) Pantoyl-taurine Diet

Introduction

Method

Result

(B) Synthetic Diet

Introduction

Method (a) Effect of a Completely Pantothenic acid Deficient Diet

Result

Method (b) Effect of a Partially Pantothenic acid Deficient Diet

Result

(C) The Influence of Pantothenic acid (Bepanthen-Salbe) Ointment on Healing of Skin Wounds

Method

Result

The Influence of Pantothenic Acid on Healing of Skin Wounds in Rats.

A) Pantoyl-taurine Diet.

Introduction. Since Pantothenic Acid is present in a large majority of foodstuffs, it is more difficult to obtain a diet free of the vitamin than in the Ascorbic Acid experiments. The usual method is to prepare a synthetic diet which is adequate in all respects apart from the substance in question. This, however, is rather a time-consuming job and an attempt was made at first to produce a deficient diet by the use of a compound - Pantoyl-taurine - which was known to inhibit growth of bacteria needing pantothenate.

Snell (1943) stated that Pantoyl-taurine, the physiologically inactive sulphonic acid derivative of Pantothenic Acid, interferes with the metabolism of Pantothenic Acid, by lactic acid bacteria and yeast, apparently by blocking the essential Pantothenic Acid away from its site of action. This is a form of growth inhibition in which the effect of one component may be nullified by the addition of a sufficient quantity of another, each compound being closely related. Barnett and Robinson (1942) in their investigation on the biological activity of Pantothenic Acid analogues found that the degree of inhibition by Pantoyl-taurine depends on the

amount of acid present and is reversed in a regular manner by addition of the acid. McIlwain and Hawking (1943) found that, although Pantoyl-taurine was rapidly excreted by rats, the ratio of Pantoyl-taurine : Pantothenate concentrations in their blood could be kept above the range of in vitro action for most of the day by frequent subsutaneous doses, to which there was no intolerance.

Snell (1943) subsequently claimed that rats fed with a certain amount of Pantoyl-taurine soon showed signs of Pantothenic Acid deficiency. He discovered, however, that Pantoyl-taurine is relatively inactive on single oral or intraperitoneal administration to rats or mice, no effects being noted from doses as high as two gm./kilo of body weight. Nevertheless after three to four weeks on daily oral administration of Pantoyl-taurine at a dose level of 200 gm./kilo of body weight, growth in standard strains of mice ceased, hair roughened and porphyrin deposits appeared on whiskers. Purina dog chow was the diet used which, in the absence of Pantoyl-taurine, contained adequate Pantothenic acid for the mice.

Method. A pilot experiment was carried out using two rats, Control and Principal, the weight of each at the beginning being 195 gm. Dog chow being unobtainable at present, a special brand of dog food was used for the basal diet.

According to Snell's estimation, the amount of Pantoyl-taurine required per kilo of body weight is 200 gm. Thus, since the weight of the rat at the beginning of the experiment was 195 gm., 39 gm. were given to the Principal each day. This dose was freshly dissolved in water each day and administered by pipette every 24 hours for one month to the Principal. The Control only received the basal diet.

Result. No external symptoms of Pantothenic acid deficiency were observed in the Principal at the end of the period and there was a steady rise in weight and growth. The loss of weight at first may be attributed to the change of food and it was several days before the rats became accustomed to the dog food. The Control rat's progress was normal and Figure 18 shows the similarity in weight curves. It thus appears that daily administration of Pantoyl-taurine to rats, receiving Pantothenic acid in the diet, does not influence significantly the growth of the animals, and it was thus found necessary to introduce a synthetic diet.

B) Synthetic Diet.

Introduction. Several synthetic diets have been described for such experiments - Woolley (1941); Unna and Richards (1942); Wainwright and Nelson (1945) - but the simplest and most effective diet is the one used at the Lister Institute - Bacon and Jenkins (1943) - and this, after several unsuccessful pilot experiments with other diets, was used here. Two

Figure 18

Figure 18 - Graph showing the weight curves of Control rat, receiving basal diet only, and Principal rat, with 39 gm. Pantoyl-taurine added to diet daily. Dog food was used for the basal diet. Slight decrease at first in weights of both animals probably due to adjustment to diet as the graph shows a steady rise after a week on the new diet.

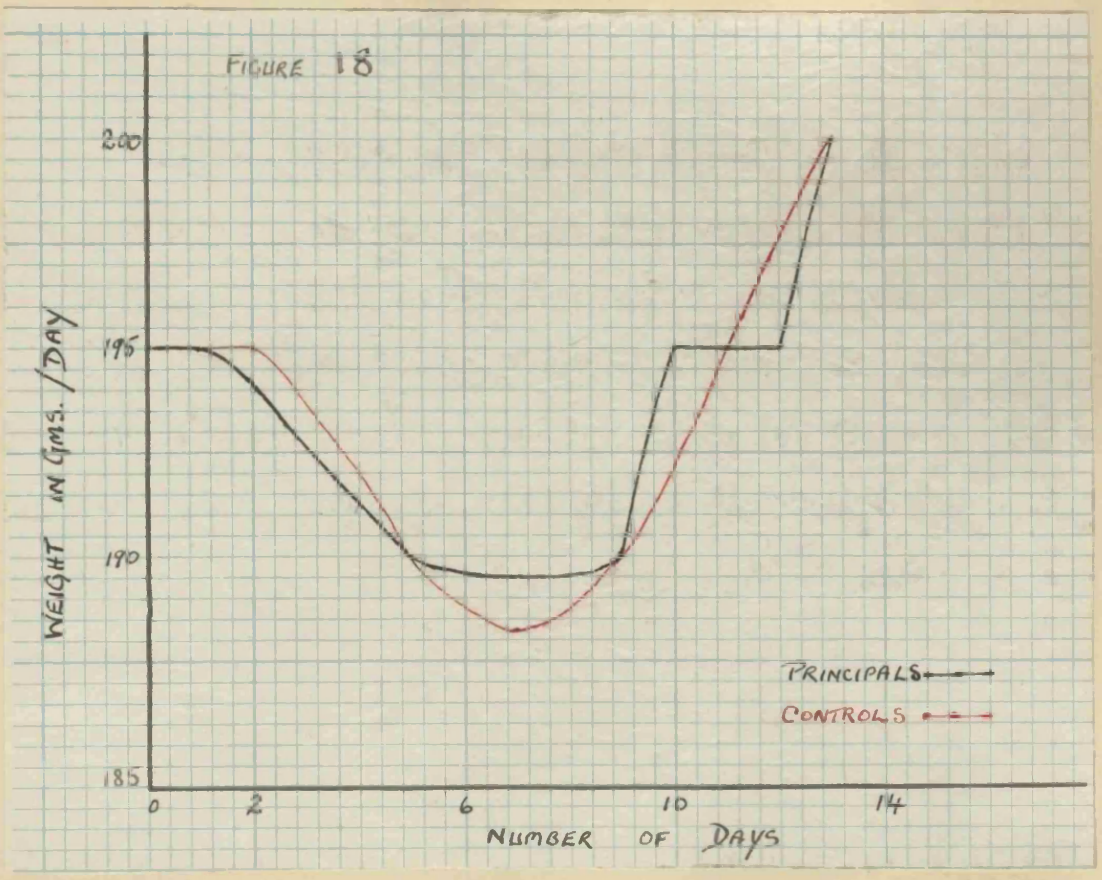
FIGURE 18
200
195
190
185
WEIGHT IN GMS. / DAY
0
2
6
10
14
NUMBER OF DAYS
PRINCIPALS
CONTROLS

FIGURE 18

solutions of mixed vitamins are given in addition to the basal diet of Sucrose, Casein (vitamin-free,) Arachis oil, Salts and Lard.

Solution A consists of

30 µg. Aneurin
30 µg. Pyridoxine
50 µg. Riboflavin
} in 2 c.c. solution daily per rat.

Solution B consists of

30 mg. Choline
10 mg. Nicotinic Acid
10 mg. Inositol
} Per c.c. solution of which 2 drops given daily per rat.

2 drops of Cod Liver Oil (Vitamins A and D) given daily per rat.

Method. a) Effect of a completely Pantothenic Acid deficient diet on healing of skin wounds.

The usual procedure in an investigation on Pantothenic Acid deficiency is to place the rats on experimental diets from the day of weaning, day 21. Consequently 10 Principals and 10 Controls were used for this experiment at three weeks of age. Each rat received 6 gm. of the basal diet, to which were added Solutions A and B and Cod Liver Oil. The Controls in addition were given 25 µg Pantothenic Acid daily in ½ c.c. water while the Principals received none. The animals were

fed this diet for four weeks before the operation. Wounds were made on both thighs in the usual manner and left untouched. Result. All animals were weighed twice per week and it was not long before the Principals began to show signs of retarded growth and weight steadily diminished - Fig. 19. After approximately three weeks on this diet, the characteristic symptoms of the deficiency gradually appeared in the Principals. One of the most noticeable signs was alopecia which developed across the shoulder blades and down both sides - contrast Control and Principal Figs. 20 and 21.

Achromotrichia was more difficult to observe as the rats were of the piebald strain, but in certain cases greying of the black patches of hair was quite striking. Porphyrin deposits were frequently perceived on the nose and whiskers and, as the deficiency became more severe, the eyes became affected and were often unable to open. The most acute symptoms appeared after 45 days on the diet and at this stage many of the Principals died.

The result of the thigh wounds was more or less to be expected considering the state of health of the Principals. The Controls healed in approximately 12 Days, while the Principals at this stage showed no signs of repair. Haemorrhages were not so noticeable as was found in Ascorbic Acid deficiency, but scab formation took place slowly and the

Figure 19.

Figure 19 - graph showing the weight curves of Control rat, receiving 25 ug. Pantothenic acid daily, and Principal rat, with no Pantothenic acid in the diet. Each rat received 6 gm. synthetic diet daily. The Control rat showed a normal growth curve with a steady increase in weight. On the other hand, the Principal's weight increased for a short time, approximately two weeks, but thereafter diminished rapidly.

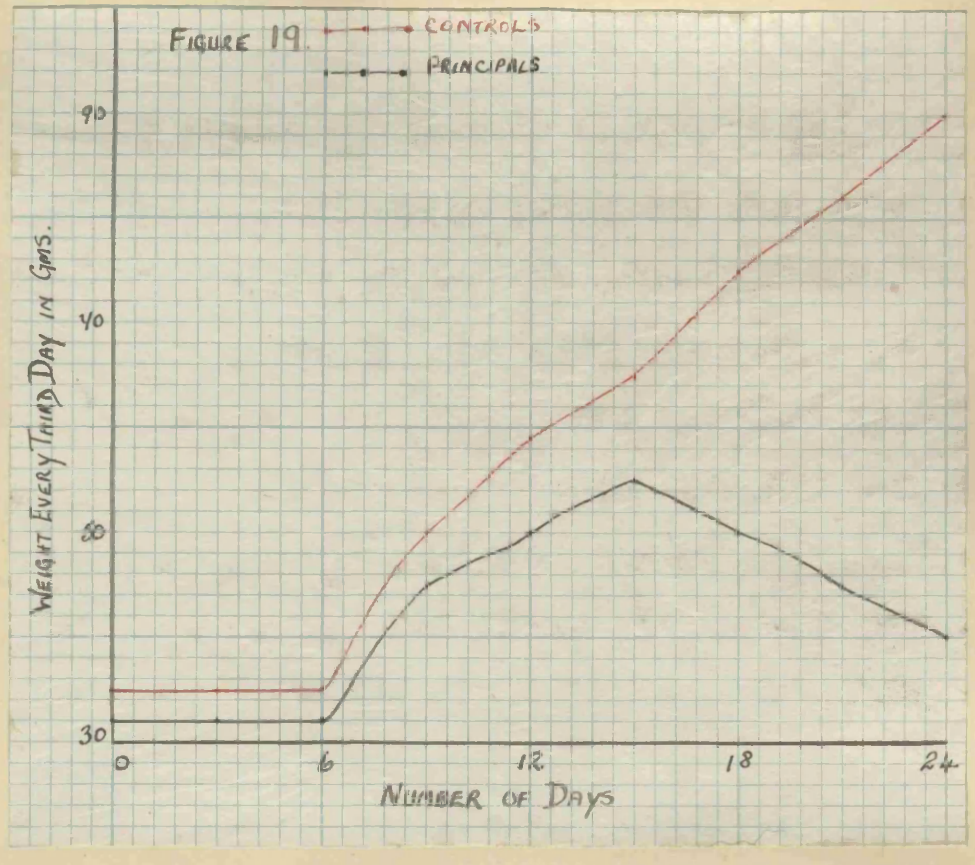
FIGURE 19.
CONTROLS
PRINCIPALS
90
40
30
0
6
12
18
24
WEIGHT EVERY THIRD DAY IN GMS.
NUMBER OF DAYS

FIGURE 19

Figure 20 and 21

Figure 20 shows a picture of a Control rat receiving sufficient Pantothenic acid in its diet - 25 ug. It is the normal size of a rat of approximately six weeks old and its skin and fur are perfectly healthy.

Figure 21 shows a picture of a Principal rat on a completely Pantothenic acid deficient diet. This presents a startling contrast to the above picture of the Control rat. The animal is very much smaller in size and weight. The hair across shoulder blades and down the sides is scanty and the black patches are beginning to turn grey. The rat in this picture is under ether anaesthesia - the reason for its eyes being closed - but in actual fact the eyelids have become slightly affected and the animal has some difficulty in opening its eyes properly.

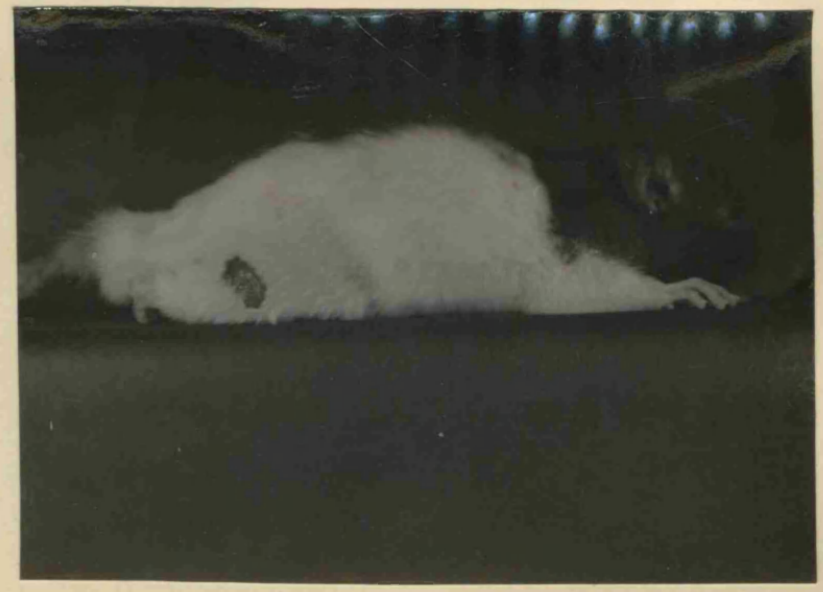

FIGURE 20

FIGURE 21

wounds did not diminish in size for some time. Unfortunately, the deficient diet proved to be too severe and the Principals started to die before complete healing had taken place. It was quite obvious, however, that a completely deficient diet had a most serious delaying effect on healing of skin wounds, although the actual healing time to complete healing could not be recorded due to the animals not surviving.

Method. b) Effect of a partially Pantothenic Acid Deficient diet on healing of skin wounds.

With the knowledge that an absolutely deficient diet can retard healing so extremely, it seemed desirable to find out if a partially deficient diet could be introduced in the rat, so that it would live long enough for the healing process to be observed to the end point and to discover if there was a similar retardation as in the previous experiment.

To find out the correct suboptimal dose for this partial deficient state, it was necessary at first to feed the Principals varying doses from 2.5 µg. - 12 µg. of calcium pantothenate daily. Only the animals receiving 12 ug. calcium pantothenate daily survived, the lower doses again causing death after several weeks on the diet. Thus, in this experiment, 10 Principals were fed 12 µg. pantothenate daily along with the synthetic diet and the Controls received the same dose of 25 µg. as before. The animals were kept

on this diet for two weeks before the operation, in which both thighs of Controls and Principals were wounded in the usual way.

Result. The weights of Controls and Principals were recorded on alternate days and as can be seen from Fig. 22 the Principals increased for a certain period and then remained stationary. There was no drastic terminal fall of weight followed by death, as noted in the completely deficient experiment, but growth was definitely retarded. No external symptoms of deficiency were observed, such as alopecia, porphyrin deposits etc., and the only sign of a deficient state was the inferior growth response in the Principals.

There was not the striking difference in the healing times of Principals and Controls as previously found in the last experiment, but it was quite evident that the Principals once again showed a delay in healing. In an average of 12 days all wounds of Controls had healed while the Principals averaged 16 days. This difference in the healing period was proved to be significant. - Table 13.

This result undoubtedly confirms the fact that even partial Pantothenic Acid deficiency bears an important relationship to indolent healing. If this vitamin is so necessary to the living cell it may be true that excess of it will be able to stimulate growth and repair, and with this view in mind the following pilot experiment has been

Figure 22

Figure 22 - Graph showing the weight curves of a Control, receiving 25 ug. Pantothenic acid daily in addition to the basal diet, and a Principal rat, receiving 12 ug. Pantothenic acid daily thus keeping the animal on a partially deficient diet. The Control as usual showed a steady increase in weight while the Principal showed a gradual rise for a few days and then remained stationary. The Principal's weight remained like this until the end of the experiment, no terminal fall of weight being observed as was found in the completely deficient experiment.

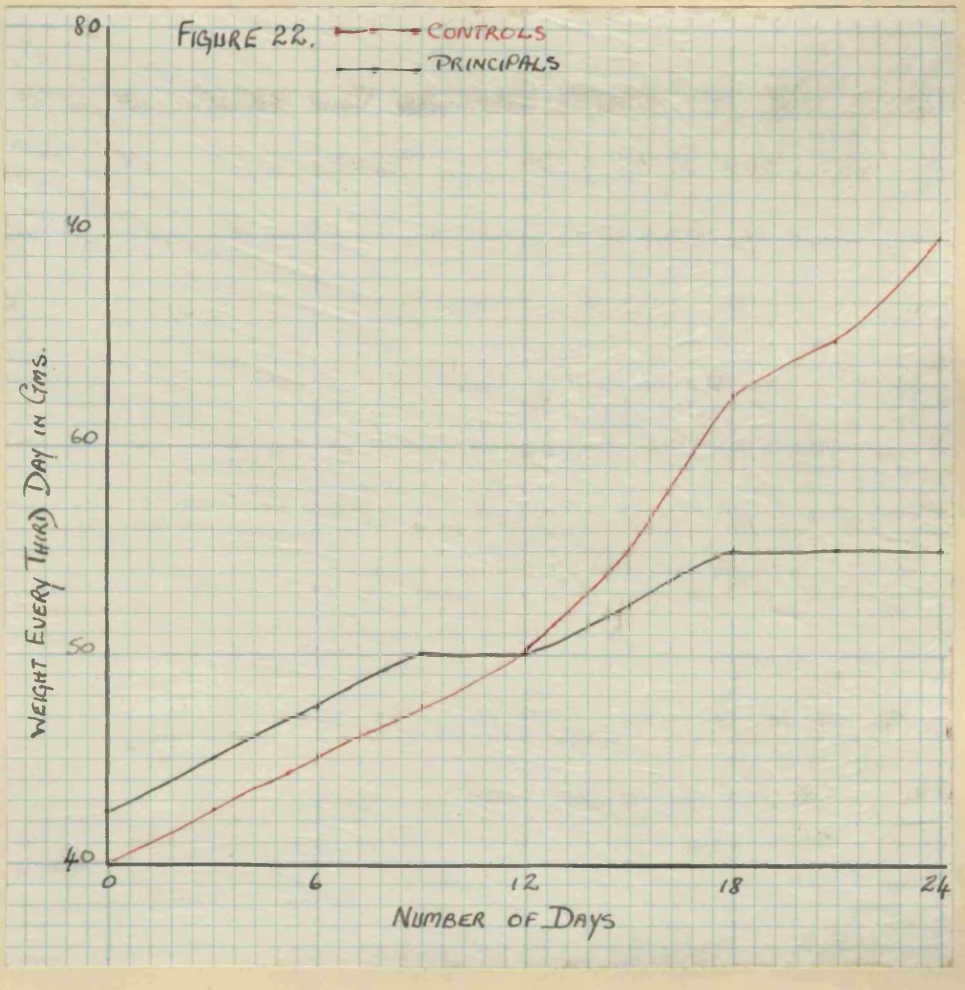
FIGURE 22.
CONTROLS
PRINCIPALS
80
40
60
50
40
WEIGHT EVERY THIRD DAY IN GMS.
0
6
12
18
24
NUMBER OF DAYS

FIGURE 22

TABLE 13.

Effect of a partially deficient Pantothenic acid diet on healing of skin wounds.

Control:- Standard synthetic diet, with 25 µg. Pantothenic acid daily. 10 rats with bilateral thigh wounds - 20 wounds which healed in 12 days.

Principal:- Standard synthetic diet, with 12 µg. Pantothenic acid daily. 10 rats with bilateral thigh wounds - 20 wounds which healed in 16.15 days.

No. of Wounds	Mean Period required for healing		Difference between Controls and Principals DAYS
	Days	± S.E. of mean	
20 C	12	± 0.33	4.15
20 P	16.15	± 0.985	

C = Controls
P = Principals

$$\frac{m_p - m_c}{\sqrt{E_p^2 + E_c^2}} = \frac{4.15}{1.03} = 4.03$$

This result is statistically significant and proves that Partial Pantothenic acid deficiency bears an important relationship to indolent healing.

carried out.

C) Influence of Pantothenic Acid (Bepanthen-Salbe) ointment on healing of skin wounds.

Method.

Four rats were used in this experiment, two Controls and two Principals and the animals were given the same diet as in the previous experiment i.e. in addition to the basal diet the Controls received 25 µg. pantothenate daily and the Principals 12 µg. After three weeks on this diet the animals were wounded in both thighs as before. This time one Control had both wounds covered with Bepanthen-Salbe while the other Control's wounds were left untouched. Similarly one Principal had the ointment put on both wounds and the other Principal left alone. This Salbe was put on the wounds each day until complete epithelialisation had taken place.

Result. The result of this direct application of Pantothenic Acid to skin wounds was most interesting. Both animals which had the ointment applied to their wounds healed in a shorter time than the untouched rats. There was not a wide difference between the groups but quite important enough for further work to be carried out.

Control with ointment.....10 days to heal.

Control with no ointment.....12 days to heal.

Principal with ointment.....13 days to heal.

Principal with no ointment.....17 days to heal.

No statistical analysis can be worked out with this pilot experiment but even without this work it is quite obvious that the Principals shows a significant acceleration while the Controls results are of great interest with regard to wound healing in the clinic.

GENERAL SUMMARY.

1. A brief historical introduction is given. It deals with the general features of wound healing in which epithelial participation is the dominant feature. The influences of such factors as Size, Age and Diet on epithelial regeneration are briefly mentioned and, in general, the effect of vitamin deficiencies. The chemistry and physiological importance of Ascorbic Acid and of Pantothenic acid, with special regard to wound healing, are discussed in fuller detail. This work is chiefly concerned with the role of these two in wound healing.
2. In Section (A) the Influence of Ascorbic acid on Healing of Skin Wounds in Rats is discussed. The importance of Ascorbic acid in healing of incised wounds has been well demonstrated by previous workers, but there are no references to the effect of Ascorbic acid on epithelial wounds of large superficial area involving little or no connective tissue.

   Bilateral skin wounds, involving only epidermis and cutis vera, were made on the thighs of rats.
   a) Ascorbic acid was administered orally to a Control Group, while a Principal group was kept on a diet free from Ascorbic acid. Since rats are able to synthesise Ascorbic acid, the Controls were receiving a "luxus" consumption. Results showed that oral administration of excess Ascorbic acid does not influence the rate of healing of skin wounds in rats.

b) Ascorbic acid powder was applied directly to skin wounds in the Principal group of rats, while the Control Group had their wounds left untouched. The diet consisted of only rat-cake nuts and water in all cases. Results showed that the Principals took 7 days longer to heal than the Controls. As even moderate acidity has a definite retarding effect on wound healing, there seemed little doubt that this was the cause of the delay, as the pH of the Ascorbic acid powder insolution was almost 4.

c) By using a "buffered" solution of Ascorbic acid, it was shown that the previous delaying effect was due to pH. Two c.c. (100 mg./c.c.) sodium ascorbate was mixed with 18 c.c. Ringer giving a solution of pH approximately 6.6 and solute concentration of 1 per cent. No difference was found in the rate of healing between the series with Ascorbic acid and the group without the acid. A stronger solution of sodium ascorbate was used with similar results, which proved conclusively that pH had been the cause of the delay in the previous experiment.

Thus excess or "Luxus" Ascorbic acid intake has no effect on the healing of skin wounds in rats either when given by mouth or applied locally.

3. Second wounds were inflicted at the same site as the initial wounds one month after the latter had healed. A significant acceleration was observed in the healing time of the second wounds. Other workers have studied this problem.

They inflicted the second wounds during the intermediate stage of healing of the initial wounds. The rate of healing was accelerated, due possibly to the presence of a "wound hormone". This "wound hormone" was said to be fugitive and to disappear rapidly after healing of the initial wound. It would appear from the present work, however, that these hormones remain during the entire period of healing and are still active some time after disappearance of the initial injury.

4. In Section (B) the Influence of Ascorbic Acid on Healing of Skin Wounds in Guinea Pigs is investigated. Bilateral skin wounds were made on the thighs.

a) Oral administration - 5 mg. given to Control group daily and ½ mg. to Principals on alternate days. A striking difference in the appearance of wounds was observed. When the Controls healed, in 16 days, the Principals' wounds were still covered with a scab and they did not heal until 29 days after the operation. This result is quite distinct from the rat experiment, and it may be deduced that, when an area of skin is removed in an animal, such as the guinea pig, liable to suffer from Ascorbic acid deficiency, administration of Ascorbic acid is indicated.

b) A solution of sodium ascorbate was directly applied to the skin wounds in two groups of guinea pigs - one group receiving 5 mg. Ascorbic acid daily by mouth and the other

½ mg. on alternate days by mouth. Two other groups, receiving the same oral doses, had their wounds treated with Ringer only. Results showed that, in the guinea pigs receiving adequate vitamin C by mouth, there was no difference in the rate of healing. In the cavies on a minimum dose of Ascorbic acid there was a different picture. In these animals application of sodium ascorbate directly to the wounds accelerated healing by 10 days. It may be deduced from these results that Ascorbic acid in excess appears to have a negligible effect on the rate of healing of skin wounds but there is no doubt that direct application of sodium ascorbate to a skin wound in a vitamin C deficient animal accelerates healing.

c) To determine whether the action of Ascorbic acid on the healing process was local or systemic, ten Principals were given ½ mg. Ascorbic acid orally on alternate days. Each wound on the right thigh was painted daily with the ascorbic acid solution, and the left wound painted daily with Ringer. Results showed that both wounds healed in approximately the same time. The obvious answer is that Ascorbic acid is absorbed from the surface and exerts its action systemically.

5. In order to discover the action ascorbic acid has on Ear wounds, the tips of guinea pigs' ears were cut off.

This is a similar type of wound to the thigh one except the skin is rigidly fixed instead of being loosely attached to the underlying tissues. A Control group received 5 mg. Ascorbic acid daily and a Principal group received ½ mg. on alternate days. A significant delay was observed in the Principals which agrees with the previous skin experiments and proves how important Ascorbic acid is in the healing of skin wounds.

6. The Influence of Ascorbic acid on the regeneration of corneal epithelium in guinea pigs is discussed. A Control and Principal group were fed the usual oral dose of Ascorbic acid and circular abrasions were made on the cornea by a new technique. This wound affected only the epithelium. Healing was most rapid, both groups being healed in an average of 45 to 46 hours. The absence of any influence on the wounds does seem to suggest that Ascorbic acid deficiency only affects the healing of wounds when connective tissue is present and that the power of epithelial proliferation is not impaired.

7. The effect of Ascorbic acid on healing of Muco-periosteum was studied. Two groups of guinea pigs, Controls and Principals, were given the usual doses of Ascorbic acid by mouth in solution. The animals were wounded in the

diastema of the upper jaw and the front of the lower jaw. The rate of healing of the gum wounds was similar in both groups, the average period being 6 days, which was a most surprising result. It was thought that this was due to the absence of another dietary factor, and an experiment was carried out using orange juice instead of Ascorbic acid in the Controls. The Principals were given the usual dose of Ascorbic acid. No change in the previous results was observed. It appears that synthetic Ascorbic acid is just as efficient in the healing process as orange juice. This result suggests that, although vitamin C is of great important in the maintenance of the teeth, it plays an insignificant part in the repair of wounded gum tissue.

8. The influence of Pantothenic Acid on healing of skin wounds in Rats was studied. In order to obtain Pantothenic acid deficient animals two different types of diet were tried at first.

a) Pantoyl-taurine, a substance which is said to block the acid away from its site of action being a form of growth inhibition, was fed with the main diet which contained Pantothenic acid. 200 gm. Pantoyl-taurine per kilo of body weight were given to a rat for one month, but no difference in weight and growth was noticed between this rat and one fed on a normal diet of rat cake-

nuts. It thus appears that daily administration of Pantoyl-taurine to rats, receiving Pantothenic acid in the diet, does not affect the growth of the animals.

b) A synthetic diet, which contained Sucrose, Casein (vitamin-free), Oil, Salts and Lard and a solution of mixed vitamins, was successfully fed to rats. Control group, in addition to the basal diet, were given 25 µg. Pantothenic acid daily. Principal group at first were fed a completely Pantothenic acid deficient diet, but this proved to be too severe and the rats died after several weeks on the diet. Instead the rats were given 12 µg. Pantothenic acid daily, thus being kept on a partially deficient diet. The animals were kept on this diet for two weeks before being wounded on both thighs. Results showed that even partial Pantothenic acid deficiency bears an important relationship to indolent healing as the Principals had a significant delay in healing.

9. Pantothenic acid (Bepanthen-Salbe) ointment was applied directly to skin wounds in a Control and Principal receiving the same oral diet as above. Another Control and Principal were only given the oral diet of Pantothenic acid, their wounds being left untouched. Both animals which had ointment applied to their wounds healed in a shorter time than the untouched rats, the Principals showing a significant acceleration. As this was only a pilot experiment no statistics can be given but the results are quite important enough for further work to be carried out

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